

Thin excuses for French nuclear tests

The government of France has given thin excuses for its weapons tests; the greatest danger lies in the precedents these tests will provide for the other black sheep who may want to join the planned Comprehensive Test-Ban Treaty.

St AUGUSTINE's prayer, "Lord, make me chaste, but not yet!", applies to France. The international row over the French proposal to test a series of nuclear weapons in the Pacific is inevitable and proper, but the grounds for opposing the testing programme should be clear — and clearer than they have been to the crowds gathered to make noise outside French embassies in the past few weeks. Importantly, this is not an environmental issue in the ordinary meaning of that term. The tests will be underground, and past weeks will have impressed on those responsible the need than not a microcurie of radioactivity should escape from them. It is also important that France is not alone in earning censure; China carried out a nuclear test a few weeks ago without attracting crowds outside its embassies. The real objection to what China and France are about is that they provide awkward precedents for what may happen in the years that follow the signature next year of a Comprehensive Test-Ban Treaty (CTB).

True to form, China has given no explanation of its motives for continued testing. Under pressure, President Jacques Chirac has given two excuses: the tests, he says, are necessary to verify that weapons in the existing stockpile will still explode after lying on the shelf for some time, and it is also necessary to collect data for the development of the computer codes needed for the design of weapons in the future. (Chirac notes wistfully that six of the eight planned tests would not be required if only the United States shared information as freely with France as with Britain, without reference to the Ottawa agreement of 1943 by which Britain and the United States agreed a perpetual partnership in the exploitation of nuclear energy, and to the congressional laws by which the US administration is prohibited from sharing military nuclear technology otherwise.) Both explanations are thin, to say the least. The worry is that, if France can make them now, it (or some other would-be nuclear power) may choose to make them after the CTB comes into force.

The need to collect data for computer codes is overstated. For exactly fifty years, as it happens, the computer modelling of nuclear explosions has been one of the best supported technical art forms. For many years, the United States restricted the export of Cray computers as jealously as if they were fissile material just because of their usefulness in simulating nuclear explosions, but that horse has long since bolted. It is unthinkable that French weapons

designers have only lately woken up to the utility of computer codes in their craft, or that past tests have failed to yield the data by which the necessary parameters can be determined. Either Chirac has been hoodwinked by his technical people, or he has decided that a show of Gaullist intransigence would be appropriate at the outset of his presidency.

The business of the ageing of nuclear weapons raises a different issue. Fission bombs have as components neutron sources built around relatively short-lived radioactive elements such as protoactinium, which evidently must be renewed from time to time. Similarly, hydrogen bombs use tritium, with a half-life of a dozen years or so, which must also be refreshed. But none of that demands great technical insight. The erosion with the passage of time of the military's confidence in a nuclear stockpile is something else.

This is a psychological matter, but an important one. If, with a CTB in force, the military become less sure of their nuclear weapons as time passes, they will be less inclined to rely on them as strategic weapons, with the result that they will inch along the road to a world without nuclear weapons. That process will not be quick, and would be instantly interrupted by a violation of the treaty, but it is one of the powerful reasons why the non-nuclear signatories of the Non-Proliferation Treaty agreed in April that the promise of the CTB would cement their adherence to this cause. Chirac's generals will eventually have to live with the uncertainties the CTB will bring them. It would have been better to start them on the road right now. □

Genetics brought to book

The British House of Commons has produced a telling document on the future of genetics.

THE Mother of Parliaments, as the British parliament likes to call itself, has been well served by the House of Commons Select Committee on Science and Technology, whose report on human genetics is published this week (see page 202). The committee's conclusions are panic-free, the arguments by which it reaches them are intelligent, its findings are plainly based on a huge amount of work and it repeatedly emphasizes that its capacity to foretell the future is hampered by the speed with which



knowledge of human genetics is being enlarged. There are only two flies in the ointment: a document of 164 pages that deserves to be widely read costs £22.50 from the government printer, while parliamentary precedent suggests that the committee itself may go out of business now that the Office of Science and Technology has thoughtlessly been made a branch of the Department of Trade and Industry (when there will be a row).

In three respects, the committee goes further than others have recently done. On the patenting of genes and parts of genes, the committee correctly concludes that what matters is not legalistic argument on whether a cDNA copy of a gene without its introns is an artefact in the sense of patent law, but whether it has utility in that context. Sequence tags should no more be patentable without a specified application than entire genes, the law that allows the use of patented techniques without royalty in bona fide research (shades of the use of the polymerase chain reaction in academic laboratories) should be clarified and the danger that patent offices, relying on precedents, will tend to grant over-broad patents in quickly moving fields — all these are cogent arguments to make. The committee is right to conclude that the European Union is unlikely to draft a useful directive in time to meet urgent need under these and other headings. (The European Parliament scuppered this year's approximation.) Maybe the British government should reconcile itself to an annual updating of its Patents Act 1977.

Employment and insurance are the other traditional hot potatoes; genetic diagnosis can in each case proscribe individual rights. On the former, the committee draws a clear line. When and if there is reason to believe that people with specified genetic constitutions are at risk from the work they do, or are a risk to others, genetic screening should be allowed for those conditions only. That is fair. On insurance, the committee notes that British insurance companies now require disclosure of genetic tests already carried out, but not further testing. It also recognizes that that state of affairs will not last long. The committee would give the insurance industry a year in which to put forward constructive proposals or face the hazard of legislation. It would have been more helpful if the committee had said what legislation it has in mind.

On regulation, the committee's chief proposal is that there should be a statutory Human Genetics Commission, with powers ranging from the approval of genetic screening programmes and the licensing of private companies offering such services to the provision of advice on the regulation of the insurance industry and the amendment of patent law. In the committee's vision, existing arrangements for gene therapy would be subsumed. That is not just another quango, but a quango and a half. All the tasks spelled out are urgent. The first need is that they should be done. The second is that they should be done carefully as well as quickly. The committee might have made the point (but does not) that the specified tasks span the interests of several departments. Against its instincts (quangos are unpopular), the government should listen. □

Spare the messenger!

Demands for censorship of the Internet are both impracticable and unwise.

THERE is an unrefereed tale that an election poster proclaiming "Public Meeting: How Labour [or Tories] will govern" was defaced by some wag who had appended "Next Week: How to nail jelly to the ceiling". Those who wish to curb activities on the Internet must reconcile themselves to similar frustrations. That is why users and service providers in Britain should welcome the eminently practical plan of the Lord Chancellor, Lord Mackay of Clashfern, to publish in the next few days draft legislation to regulate one possible abuse: defamation. Mackay's Defamation Bill would protect providers of online services from legal action over defamatory messages disseminated under their aegis. It should thus deflect calls for the Internet to be policed or censored, and encourage users to be more responsible.

The Defamation Bill would not abolish defamation suits arising from electronic messages, but would protect those users, certainly the majority, who use the Internet in the normal course of business, and who have adopted a culture of excessive politeness in their electronic discourse. But service providers will be the chief beneficiaries. It would be wrong for a small service provider to be driven to bankruptcy, and those subscribing to that service inconvenienced, through the bruised sensibilities of a single user.

Mackay's Defamation Bill will therefore turn the spotlight onto the global gaggle of Usenet groups, the source of most grievances about the content of the Internet. The distinction between service providers and Usenet groups is important. The former are not so much publishers as conduits through which subscribers can receive a variety of online services, electronic mail included. The Usenet, by contrast, is a collective noun for the thousands of special-interest bulletin boards accessible to any Internet user through a service-provider, usually for no additional charge. The topics range from computer-buffery to hard pornography. New Usenet groups are formed every day, and old ones die out almost as quickly. Their ephemeral nature makes policing impractical.

Usenet groups are self-regulated in that the content of some of them is monitored by private enthusiasts, known as moderators, who are thus analogous to publishers or editors. Mackay's planned absolution of service providers means that Usenet moderators will be next in line for legal action, and will presumably become more scrupulous about what they let through. But if the Usenet climate then becomes more responsible, advocates of censorship will have fewer grounds for complaint. The US Senate's recent vote to make the use of the Internet for pornographic purposes a criminal offence is unlikely to survive the declared opposition of the Speaker of the House of Representatives, but a less colourful Internet would be an improvement. As things are, most Usenet postings are of no interest to socially well-adjusted people over the age of twelve. □

Accelerator-based tritium facility to win out over rival US proposals...

Washington. Hazel O'Leary, the energy secretary, is about to endorse plans to build a \$3-billion particle accelerator designed to produce tritium for US nuclear weapons. Some physicists believe the accelerator could also be used for experiments initially planned for the now-abandoned Advanced Neutron Source.

In a long-awaited decision due to be announced within the next ten days, O'Leary is expected to reject proposals to build a nuclear reactor for tritium production. But she will recommend that the use of existing nuclear power stations for this purpose should be investigated as a back-up to the accelerator.

The accelerator decision will provide a critical boost for the Los Alamos National Laboratory in New Mexico, where a four-year, \$300-million research and development programme — including the construction of a prototype — will be carried out as the project's initial stage.

The full accelerator would be built at one of the five candidate sites, including the Nevada test site and Savannah River, South Carolina, where O'Leary may make the announcement on a visit next week.

Tritium, the heaviest isotope of hydrogen, is a component of all the nuclear weapons the United States intends to retain. It decays with a half-life of 12 years, and since the

closure of a previous facility at Savannah River in 1989 the United States has used spare tritium from weapons being taken out of service to replenish its stockpile.

But the Department of Defense insists that a source must be planned now to ensure supplies after 2011. O'Leary, concerned about accusations of being soft on defence issues, has promised Congress a formal decision on the technology and the site before November. She has always favoured the accelerator proposal, as it does not involve building a nuclear reactor.

O'Leary is likely to be strongly opposed by a powerful faction in Congress that backs the reactor alternative. Her decision to back the accelerator follows her receipt of a crucial report on the economics of tritium production prepared by an independent Washington consulting firm, Putnam Hayes and Bartlett (PHB).

The unpublished report found that using an existing civil reactor — probably at Plant Vogtle, near Savannah River — would be far cheaper than either construction proposal. Industry sources say that the report has forced O'Leary to consider the civil reactor option more closely, and that before making a final decision she must decide whether to pursue the civil reactor as a back-up to the accelerator or to research both options fully in a 'dual track' approach to the problem.

The use of a civil reactor for military tritium production would conflict with the traditional separation of military and civil nuclear work in the United States, and — despite its immense cost — the construction of a brand-new facility has heavyweight political support.

O'Leary and other friends of the national laboratories, led by Senator Pete Domenici (Republican, New Mexico), want an accelerator. By contrast, a new reactor, which would cost up to \$6 billion and would also probably be built at Savannah River, is supported by Strom Thurmond (Republican, South Carolina), chair of the Senate Armed Services committee, and Floyd Spence (Republican, South Carolina), chair of the House of Representatives National Security committee.

The reactor is seen by the nuclear industry as its last and best chance of building a nuclear plant in the United States — the first for 15 years. But its supporters received a setback last month, when an attempt by Spence's committee to by-pass the Depart-

...as German reactor faces concerns

Munich. Hazel O'Leary, the US energy secretary, has told the German authorities that she would prefer to see the controversial new research reactor FRMII converted to burning low-enriched uranium (LEU) fuel, rather than highly enriched uranium as currently planned.

But the letter, which reflects O'Leary's concern about the dangers of civilian trade in bomb-grade plutonium, is being played down by the State Department, apparently keen that the United States should not be seen to interfere with the domestic decisions of another country.

O'Leary's statement was made in a letter to Paul Leventhal, president of the private US Nuclear Control Institute. In reply to an enquiry about the US response to the FRMII, O'Leary wrote on 7 July that US efforts to enter the domestic debate in Germany over the reactor would be "potentially counter-productive". But she added that the United States "had expressed to

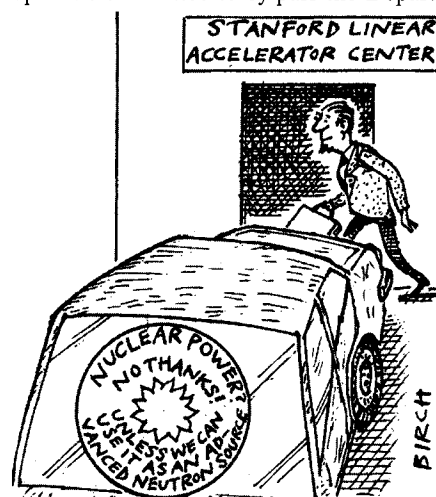
German authorities [the] hope that the FRMII will use only low enriched fuel".

O'Leary added that she had informed Gebhard Ziller, parliamentary state secretary for research, of US concern last September, and had "offered US assistance in redesigning the FRMII".

A spokesman for the Department of Energy (DoE) in Washington says the offer referred to meetings last year of German scientists with researchers from the Argonne National Laboratory who have experience in nuclear fuels of differing enrichment levels.

The DoE spokesman added that the discussions between Ziller and O'Leary had been informal, but also admitted that the State Department had not been happy with O'Leary's letter to Leventhal.

Gert von Hassel, a spokesman for the FRMII reactor programme, says that O'Leary's letter is not an "official diplomatic protest". Despite continuing protest, construction of the reactor is due to start next year. **Alison Abbott**



ment of Energy (DOE) and spend \$100 million on the reactor was attacked as an 'earmark', and defeated on the House floor.

O'Leary was briefed last week on the PHB report, which argues billions of dollars could be saved if the government either paid an electricity utility company to make the tritium in existing plant or purchased an existing power plant outright to produce it.

At the same time, a number of observers, including supporters of the nuclear weapons programme, believe that the tritium facility is entirely unnecessary. According to one former senior official of the Bush administration, there is "no national security need" for the facility, as present stocks will arm thousands of weapons for decades to come, and extra supplies can be obtained from ►

►civil power plant in an emergency.

Anti-nuclear groups are more direct. "This is 75 per cent a 'pork barrel' issue — particularly in South Carolina, where they are scrambling for any jobs programme they can find," says Tom Clements of Greenpeace.

The accelerator would use a high-power version of LANSCE, the existing linear proton accelerator at Los Alamos, to drive protons into a target, producing neutrons which in turn would convert lithium-3 gas into tritium. "The accelerator has a lower capital cost than the reactors, but higher operating costs," says Paul Lisowski, head of the Accelerator Production of Tritium (APT) office at Los Alamos, who admits it would consume a vast amount of electricity — 350 MW, enough for a city of a third of a million people.

Critics say the technology is untried. But reviews by various groups, including a Jason panel chaired by Sidney Drell, the Stanford physicist, have said that it will work. According to Lisowski, the research phase would establish the best target materials, and use a prototype of the front section of the accelerator to iron out any problems with dispersal of such a high-power beam.

Physicists, who were recently deprived of a proposed advanced neutron source of their own (see *Nature* 373, 460; 1995), are now likely to pursue changes in the APT project that would allow it to be used for experiments.

Earlier this year, for example, Burt Richter, director of the Stanford Linear Accelerator Center (SLAC), suggested this idea to Martha Krebs and Vic Reis, the assistant secretaries in charge of energy research and nuclear weapons.

In a letter to the two officials, Richter argued that changes costing "a few hundred million dollars" could provide the United States with "the world's premier spallation source" of neutrons. He also pointed out that, subject to future arms control agreements, tritium may not be needed in the quantities envisaged.

Krebs rejected Richter's plan on both technical and financial grounds. But laboratory officials say that O'Leary's decision to proceed with the accelerator may revive it, and observers feel that the DOE's real problem is its historical aversion to joint civil-military facilities. **Colin Macilwain**

UK Parliamentary panel calls for human genetics authority

London. In a wide-ranging report that is likely to have a major impact on government policies in both Britain and elsewhere, a committee of the UK House of Commons has recommended the creation of a statutory body with broad responsibilities to regulate the applications of human genetics.

The activities of such a body — which it suggests should be called the Human Genetics Commission — would range from advising local ethics committee on research involving genetic screening, to regulating companies offering genetic services.

The committee has also proposed reforms in the way the patent system is applied to genetic information which would, it hopes, maintain the benefits of the system while limiting its potential for abuse.

In particular, while accepting that human gene sequences should remain patentable, it suggests that patent protection should be restricted to a particular application of the sequence — and that the discoverer of a separate application should be eligible for a separate patent.

The recommendations have emerged from an intense eight-month study by the House of Commons Select Committee on Science and Technology, which attracted 161 submissions, and involved over 12 public hearings.

Sir Giles Shaw, Conservative Member of Parliament for Pudsey and chairman of the committee, says the recommendation to set up a statutory genetics authority was a further step along a path that had already involved the creation of advisory bodies such as the Gene Therapy Advisory Committee.

"The scale of the issue raised by the application of genetic science, ranging from questions about patents to the behaviour of

the insurance industry, is, in our judgement, sufficient to warrant a comprehensive regulatory system," Shaw said this week.

The report itself points out that Virginia Bottomley, the former health secretary, recently promised that the government would consult widely on the setting up of a non-statutory committee (see *Nature* 375, 714; 1995). But it adds: "we consider that such consultation has been carried out in the course of this enquiry".

It points out that a "substantial" number of the individuals and organizations that it had consulted supported calls for a regulatory body not only to cover genetic screening — as proposed in an earlier report from the Nuffield Council on Bioethics — but "with wider terms of reference to oversee developments in genetics science."

Reflecting the broad spectrum of political views among the committee's 11 members, concern over the potential misuse of genetic information by insurance companies is tempered by reluctance to impose rigid rules of behaviour. Thus the select committee would give the insurance industry a year in which to propose a form of regulation acceptable to Parliament; only if it fails to do so would a solution be sought by legislation.

But merely highlighting such concern will reinforce calls from pressure groups for government action. Already the Genetics Interest Group, for example, which represents a coalition of medical action groups, has announced plans to use the report to back an amendment to disability legislation currently being debated in the House of Lords; this would give the government powers to act against those found guilty of "genetic discrimination" in employment.

The committee comes down firmly against patents on fragments of genes or on genes of no known function. It also insists that the principle of the exclusion from patentability on the grounds of morality — as stated in the European Patent Convention — should remain.

Its proposed compromise on individual gene sequences is that a combination of a gene and a known, novel and unobvious utility should be patentable "in the context of that utility" — while at the same time "a combination of the same gene and a further novel utility should also be patentable."

Such a formula, committee members hope, both acknowledges the validity of gene patents and restricts the breadth of their potential application, and will thus be attractive to those seeking to resolve conflict. "I hope our report will reassure those colleagues who remain unhappy about the issue of gene patents," says committee member Lynne Jones. **David Dickson**



Shaw: Issues warrant a regulatory system.

FDA approval allows gene therapy in Japan

Tokyo. The US Food and Drug Administration (FDA) has opened the way for the first clinical application of gene therapy in Japan. Last week, the agency approved the export to Japan of a retroviral vector developed by the US National Institutes of Health and the company Genetic Therapy Inc. (GTI).

Supplies of the vector are due to be shipped this week, and will be used a few days after arrival by a group at Hokkaido

University to treat a 4-year-old boy suffering from adenosine deaminase (ADA) deficiency, an enzyme deficiency that weakens the immune system.

The Japanese government approved this first application of gene therapy in February. But the Hokkaido group has been almost entirely dependent on US technology for the technique, and had to await FDA approval for the export of GTI's product.

David Swinbanks

Saturn mission feels Congress' frustration

Washington. A congressional bid to cancel the planned international Cassini mission to Saturn, as well as to eliminate three of the US National Aeronautics and Space Administration (NASA)'s 11 centres, has touched off a storm of protest both in Europe and in the US Congress.

The reaction was so strong that the plan may well be dropped when the full committee meets. But the space agency is not yet home and dry, as Republican budget-cutters in Congress continue to search for major programmes to eliminate.

The bid came from the House appropriations subcommittee that funds not only NASA but also the Environmental Protection Agency (EPA), the National Science Foundation and a miscellany of other government departments. The subcommittee voted on 10 July to cut NASA's 1996 budget request by 5 per cent, to \$13.54 billion.

The space shuttle, the space station, and the Earth Observing System (EOS) would all be funded in full. But the subcommittee proposed cancelling both the 1997 Cassini mission to orbit Saturn, a mission being planned jointly with the European Space Agency (ESA) and the Italian space agency (ASI), and the proposed Gravity Probe-B relativity experiment.

It also recommended delaying two infrared astronomy projects, SOFIA and SIRTf, and — most radical of all — closing down the Goddard Space Flight Center in Maryland, the Marshall Space Flight Center in Alabama and the Langley Research Cen-

ter in Virginia. Transferring their missions to other NASA centres would save some \$130 million a year, the subcommittee suggested.

Howls of protest were immediately heard from congressional representatives of states where the threatened centres are located,

this ridiculous suggestion goes in its proper place — the trash." Both Mikulski and Shelby sit on the Senate appropriations panel that oversees NASA funding.

A game of bluff between Goldin and Lewis seems to have been partly to blame for the subcommittee's action. Lewis had asked Goldin several times to recommend cuts in the agency's Mission to Planet Earth (which includes EOS). But Goldin had refused, steadfastly standing by the Clinton administration's budget request. Lewis said before the subcommittee vote he was left with no choice but to propose his own cuts as a "warning shot" to the agency.

The subcommittee's recommendations are not being taken too seriously in Washington. One house aide called the proposal a "non-starter", adding that the decision was motivated more by politics than a desire to shape a meaningful NASA budget. (Much of the work from the Goddard, Marshall and Langley centres, for example, would be transferred to centres in California, Lewis's home state).

Space programme observers see little chance of Goddard, the agency's lead science centre, closing down, particularly with Mikulski defending it. As for Cassini, most of the spacecraft development money — \$1.1 billion out of a projected \$1.4 billion — has already been spent, and supporters say it would be foolish to kill the project now. Terminating Cassini would also have a chilling effect on delicate negotiations about Europe's participation in NASA's space station project (see box, left). That argument alone may ensure the project's survival.

But the vote still took NASA — and some congressional members — by surprise. It also provoked the House Science committee, chaired by Robert Walker (Republican, Pennsylvania), to speed up work on its own authorizing legislation for NASA's 1996 budget. This was partly, in Walker's words, to "offer some clear alternatives to the appropriators before [the NASA funding bill] goes to the House floor".

Walker's bill is expected to be completed this week, just as the whole appropriations committee considers the Lewis subcommittee's recommendation. The bill would then go to the full House late this week or next week. The Senate takes up NASA funding in August or September, and the agency's final budget is likely to be decided in a reconciliation process in late September.

The Walker committee is likely to recommend keeping Cassini on track. But it is also expected to propose deep cuts to EOS, and may recommend cancelling some or all of NASA's proposed new projects, including the New Millennium technology demonstration spacecraft and the low-budget Lunar Prospector mission to map the Moon.

Tony Reichhardt

IMAGE
UNAVAILABLE
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REASONS

Cassini: cancellation proposals upset ESA.

who called in Daniel Goldin, the administrator of NASA, for strategy meetings. Senator Barbara Mikulski (Democrat, Maryland), for example, expressed her "deep dismay and disappointment" in a letter to the subcommittee chair, Jerry Lewis (Republican, California).

Her colleague Richard Shelby (Republican, Alabama) described the vote as "ludicrous, poor policy, and complete nonsense". He added: "I will do everything in my power to see that this does not happen and that

ESA warns of blow to cooperation

Paris. The European Space Agency (ESA) last week warned that the proposal by a budget subcommittee of the US Congress to cancel the joint US-European Cassini-Huygens mission (see above) risks damaging future cooperation in space.

In particular, officials said it could have a major impact on Europe's planned participation in the international space station. If the subcommittee's recommendation is adopted by the full Congress, it would represent a "major crisis" for ESA, warns Roger Bonnet, head of science at the agency.

Bonnet adds that the committee's position "shows a complete lack of concern for proper financial management, and a failure to properly evaluate the consequences on international politics".

ESA is particularly upset at the prospect of the mission being cancelled because it has already spent \$220 million of the \$300 million direct costs of the Huygens probe, as well as

another \$200 million in general costs associated with the mission.

"We thought that the project was safe, as it is almost completed", says Bonnet. He adds that the agency is scheduled to supply the satellite to the US National Aeronautics and Space Administration next year.

Bonnet also predicts that such a situation will also make Europe even more reluctant to cooperate with the United States in future space missions. ESA officials are already upset by US vacillation over participation in joint missions, for example over its withdrawal from the Ulysses missions to the Sun, and last year the agency decided to design all future missions so that their main scientific goals could be achieved even if partners withdrew (see *Nature* **368**, 786; 1994).

Jean-Marie Luton, the director-general of ESA, has already written a letter of protest to the congressional subcommittee.

Declan Butler

EU may help fund sarcophagus over Chernobyl remains

London. Nine years after the world's biggest known nuclear accident, the European Union (EU) is deliberating whether to contribute towards a US\$1.6-billion bill for sealing off the remains of Chernobyl reactor number four.

The EU has already funded a US\$4-million feasibility study whose results were unveiled in the Ukrainian capital Kiev last week. This recommends stabilizing and dismantling the reactor's existing sarcophagus and covering the reactor's 100 tonnes of nuclear fuel and 400 kg of plutonium with an arch-shaped shelter.

The shelter would also cover hundreds of thousands of cubic metres of contaminated debris that will remain radioactive for about 10,000 years. But the project, which would take ten years to complete, has yet to find firm backers. The EU has not announced whether member states are prepared to commit further funds, while the Ukrainian government has said it is in no position to pay even part of the required costs.

The feasibility report was compiled by Alliance, a six-member multinational consortium of companies including Britain's AEA Technology, Walter Bau of Germany and SGN of France.

The report says that the reactor's lid and chimney are unstable and could collapse at any time, and warns of an additional hazard in the form of an earthquake. The region experiences earthquakes on average every 27 years of magnitude 5 on the Richter scale.

The Ukrainian government is believed to have appealed for US\$4 billion to pay for decommissioning all four Chernobyl reactors, as well as building three new power stations. But according to Greenpeace's Nuclear Research Unit, Ukraine would probably settle for US\$2 billion, but in the form of a grant rather than a repayable loan.

Kiev has so far received US\$933 million in grants for the Chernobyl plant and allied projects. Last July, an ECU100-million (\$133-million) grant was released under a EU scheme known as Technical Assistance to the Community of Independent States.

Additional sources of funds include ECU400 million in Euratom loans and a further US\$200 million in grants agreed at the Naples summit meeting of Group of Seven leaders in July last year to complete and upgrade the three new power stations to international safety levels.

Part of the money will also go towards shutting down and decommissioning Chernobyl's reactors numbers one and three by this year and 1998 respectively. The Ukrainian president has said he is committed to closing reactors one, two and three by the year 2000.

Pete O'Neill

Nuclear waste programme under fire for 'incoherence'

Paris. A 15-year programme set up by France in 1991 to explore options for the disposal of nuclear waste lacks a coherent research strategy and is already behind schedule. This is the conclusion of a commission set up to review the programme, whose report has fuelled demands from physicists outside the nuclear establishment for a greater role in developing new techniques for handling waste.

The nuclear waste programme was established by a law passed at the end of 1991. This postponed a decision on what to do with waste from France's 55 nuclear reactors until 2006, in order to allow time for further research into three approaches for disposing of such waste.

One is to develop techniques for extracting and transmuting long-lived radioactive elements from waste. Another is to study conditions for storing low-level waste on the surface. The third requires the National Agency for Nuclear Waste both to choose candidate sites for storage of high-level nuclear wastes and to construct 'rock laboratories' at two of these locations.

In a report published earlier this month, a review commission made up of six experts nominated by the Senate and National Assembly says that research into the three approaches needs to be better coordinated by the various organizations and industrial groups involved, so that it can be treated in a "coherent and open" manner.

Its complaint represents more than a request for good housekeeping. Behind the call for better coordination appears to lie growing concern that the research programme risks being compromised by the influence of the French Atomic Energy Commission (CEA) in its choice of goals.

Claude Detraz, for example, head of the particle physics department at the Centre National de la Recherche Scientifique (CNRS), says that CEA suffers from a "culture" that is too orientated towards improving existing technologies and is "reluctant to explore to new areas". Research on nuclear waste disposal "cannot be left to CEA", he says.

Detraz's claims are implicitly supported by members of the commission, who argue that France's nuclear industry tends to restrict avenues of research to those that are compatible with existing processes.

Fundamental nuclear physics is not being given a sufficient role, they argue, pointing out the need for a broader approach that pays more attention to, for example, nuclear data, simulation techniques and models, high-intensity accelerators and materials.

In particular, both Detraz and the commission argue that more attention needs to be paid to alternative, long-term options for waste disposal, such as the transmutation of long-lived waste into shorter-lived elements.

Changing this situation requires giving CNRS a bigger role in the French programme, argues Detraz. He claims that CNRS offers a "different culture" from that of CEA, as well as greater "intellectual flexibility". He also points out that CNRS also has more nuclear physicists than CEA, whose staff are mainly engineers.

But although CNRS may have the competence to study such options, its annual budget for research into nuclear waste disposal is less than FF1 million. This sum pales beside that of CEA, which spent FF225 million last year just on a programme aimed at incinerating plutonium in fast-breeder reactors.

If the commission's recommendations are taken up by the government, CNRS may gain some ground. The most likely outcome, predict observers, is that the government will demand that CNRS and CEA, together with various other agencies involved, create a formal structure to coordinate their research on disposal.

The commission also argues that the "tightness" of the proposed research timetables means that the government may have to postpone any final decisions about waste disposal until after 2006. The government will reveal its reaction to the commission's recommendations in November when it presents a progress report on the waste disposal programme to the National Assembly.

Declan Butler

Baboon/man transplant backed for HIV victim

San Francisco. Researchers seeking to use baboon bone marrow to restore the immune system of a patient with HIV have won the support of a key advisory panel to the Food and Drug Administration (FDA).

FDA officials had urged caution because of concern that xenotransplants, or cross-species transfer of tissue and organ material, could stimulate the emergence of lethal new viruses into the human

population (see *Nature* 376, 8; 1995).

But the FDA biological response modifiers advisory committee has now responded to the pleas of AIDS activists with a cautious endorsement. Members of the committee have asked for an improved consent form, a search for disease-free baboon donors and other safety measures such as tissue sample storage and monitoring of the patient for life.

S. L.

Future of AIDS research office threatened

Washington. A US congressional subcommittee has voted to eliminate the research-funding responsibilities of the National Institutes of Health (NIH)'s fledgling Office of AIDS Research (OAR). The move calls into question the future of the two-year-old office, which is responsible for co-ordinating research on AIDS.

At the same time, the committee has also recommended a 5.7 per cent increase in funding for research at NIH as a whole, including an 11 per cent increase for human genome research. But some researchers fear that the Republican-dominated Congress is seeking to cut funding for AIDS research,

while the overall budget package — which would also abolish the post of Surgeon General — is being opposed by the Clinton administration because of its cuts in social and education programmes.

The AIDS office was set up by Congress in 1993 after researchers and activists had convinced lawmakers of the need for better coordination of AIDS research. Last week's move, taken by an appropriations subcommittee in the House of Representatives, would remove funds for AIDS from William Paul, the director of OAR, and return them to the general funding stream at NIH.

"The bill removes numerous earmarks

and instructions that placed political considerations ahead of scientific decisions," said the subcommittee chairman, John Edward Porter (Republican, Illinois), as he opened the vote on the massive spending bill for the Departments of Education, Labor, and Health and Human Services.

After discussing the issue with Arnold Levine of Princeton University, the head of OAR's evaluation team, Nancy Pelosi (Democrat, California), put forward an amendment to restore OAR's budget responsibilities. But it failed to win the necessary support of the subcommittee.

"If we start setting political priorities, there is no way to know where the money will go," said Robert Livingston, chair of the full appropriations committee, who attended the vote and expressed concern about attempts to single out AIDS research for special treatment.

"Heart, lung and blood diseases account for half of the deaths in this country, yet they receive only 7.2 per cent of NIH's budget," said Livingston. "We spend \$295 per patient on cancer research, \$158 per patient on multiple sclerosis, \$93 on heart disease, \$54 on Alzheimer's, \$26 on Parkinson's — and \$36,000 per AIDS patient on research."

Under the subcommittee's bill, the NIH would receive an increase of \$642 million over its 1995 budget. This is a significant contrast to the \$9.3 billion in cuts and terminations that the bill would impose on social service programmes, including the elimination of 163 job training initiatives and 50 education programmes.

The National Cancer Institute would receive an increase of \$114 million, 5.3 per cent over 1995 levels, and the National Centre for Human Genome Research an extra \$17 million, to \$170 million.

The proposed budget increase for the NIH may be short-lived. The White House has made it clear that President Bill Clinton will not sign the bill unless at least some of the terminated programmes are reinstated.

But lobby groups keen to see the NIH budget increase, ranging from those representing patients' groups to pharmaceutical companies, are likely to put pressure on Clinton to sign. "You either take the money out of other programmes, or you take it out of NIH," says Dave Moore, of the Ad Hoc Group for Medical Research Funding, concerned that pressure to restore social programmes may mean a raid on NIH funding.

The level of AIDS research funding is not specified in the subcommittee bill. Both Porter and Livingston say this does not necessarily imply that AIDS research will be cut. But AIDS researchers believe otherwise, and have already drafted a letter of protest to Livingston.

The argument that removing the funding authority of OAR will not have any ►

Lawyers 'built tobacco smokescreen'

San Francisco. Last week, the US Food and Drug Administration (FDA) announced its conclusion that nicotine is a drug — and can therefore be legitimately regulated as such. The announcement coincided with the publication of an analysis of tobacco industry documents revealing that industry lawyers have controlled the direction and reporting of both internal and external scientific projects in order to keep knowledge of tobacco's deleterious effects hidden.

The analysis is based on confidential internal documents from Brown & Williamson Co. and British-American Tobacco Co., and was carried out by researchers at the University of California, San Francisco (UCSF), which had received them from an anonymous source.

The researchers concluded that the lawyers promoted certain research, steered the company away from other work and influenced the ways in which studies were reported, and that their goal was to influence government policy-makers, convince the public that smoking is safe, and develop ammunition for liability suits.

The documents, which UCSF fought to keep in the public domain, were reviewed in detail by UCSF researchers in the 19 July issue of the *Journal of the American Medical Association*. More than half are directly available on the Internet.

Brown & Williamson called the articles "a cherry-picking exercise", arguing that it was natural for its lawyers to be involved in defending the company's position. "Brown & Williamson's lawyers have conducted themselves appropriately," it said.

But Stanton Glantz, a professor of medicine at UCSF, says that the reports and correspondence revealed an unprecedented manipulation of science. Most remarkable, he says, was the contrast between an extremely sophisticated internal scientific effort, and what he described as the "junk" science encouraged by the company through outside grants. But he says that the papers

also reveal that, over time, company lawyers were eventually able to exert control over internal projects as well.

The documents indicate that company scientists were decades ahead of mainstream researchers in understanding nicotine addiction, cancer, passive smoke, chronic bronchitis and the physics of smoke. But correspondence between company executives and their legal advisers suggest the

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Lighting up: study claims that tobacco companies were way ahead in research.

lawyers sought to keep findings away from the scientific community and the public.

In their report, the UCSF researchers detail documents showing that tobacco company lawyers reviewed scientific manuscripts before they were published, sometimes changing the language or citations to serve the company's purposes. The report also suggests the lawyers tried to halt discussion of certain topics among scientific colleagues, warning that statements seeming to admit that smoking caused cancer or other disease would be harmful in court.

The 8,000 pages of documents include hundreds of examples of the vast difference between the internal world of tobacco industry research and the face that the companies present to the outside world. "It's our first crack in the wall to see what was really going on in these places," says Glantz, who led the months-long inquiry by five specialists into the documents' contents. **Sally Lehrman**

Sally and Richard Greenhill

► impact is "nebulous", says John Moore of the Aaron Diamond AIDS Research Center in New York, chair of OAR's vaccine research and development committee. "If that is the case, why do it?"

The letter, drafted by Moore, says that before the creation of the OAR, the funding of AIDS research at NIH "was inefficient, leading to an uncoordinated, duplicated and disorganized allocation of federal funds".

Under the eye of Levine, the OAR is reviewing this system. It will report to Congress in September, in preparation for a major overhaul of the type of AIDS research undertaken at NIH. The scientists say Congress should delay any decision about the office until the report has been submitted.

But the panel's action has not come as a surprise. "We knew Republicans didn't hold OAR in favour," says Moore. Furthermore, he points out, the influence of AIDS activists with Republicans is considerably less than that of others fighting to defend the NIH budget.

Spencer Cox, of the Treatment Action Group in New York, says that his group is particularly concerned about the prospect that control over AIDS research funding will be returned to Anthony Fauci, director of the National Institute of Allergy and Infectious Diseases which conducts about half of AIDS research at NIH.

"Paul emphasizes basic, baseline research," says Cox, claiming that this contrasts with Fauci's earlier approach when he held sway over AIDS research funding at the institute. Cox's comments suggest that the latest congressional moves have rekindled the animosity previously directed at Fauci by some members of the AIDS community.

Adrianne Appel

Serco wins physics laboratory contract

London. Ian Lang, Britain's secretary of state for trade and industry, announced last week that a five-year contract to manage Britain's National Physical Laboratory (NPL) has been awarded to a consortium led by Serco, a contract management group first created in 1929 by the US company RCA to manage its cinemas in Britain.

In a written statement to the House of Commons, Lang said that the consortium, which also includes AEA Technology and Loughborough University, will be expected to extend NPL's commercial work without compromising its status as a world centre of excellence in metrology.

Serco was bought out by its managers in 1987, and has grown rapidly as the government has privatized the management of public services. The defence industry accounts for 36 per cent of its business. But the group's other interests range from running a prison in Doncaster in the north of England to looking after 17,000 parking meters in Hong Kong. □

Major claims policy changes will 'strengthen' UK science

London. The British government found itself having to fight hard last week to justify its decision to move the Office of Science and Technology (OST) out of the Cabinet Office and into the Department of Trade and Industry (DTI).

Faced with a continuing stream of protest from both the scientific and political communities, Ian Taylor, an under-secretary of state within the DTI and given responsibility for science and technology, said that the science office was being moved "closer to the effective heart of government".

Taylor, whose rank is usually referred to as that of junior minister, also dismissed concerns that the result would inevitably be a reduced commitment to long-term, fundamental research. His remarks came shortly after John Major, the prime minister, had told the House of Commons that the purpose of the move was "to strengthen the contribution of science, engineering and technology to long-term wealth creation."

But the scientific community remains suspicious. The pressure group Save British Science (SBS), for example, stayed on the offensive. One of its co-founders, Dennis Noble of the University of Oxford, criticized the move in a speech to the Physiological Society as "disastrous", claiming that it could, if handled insensitively, "quite simply strangle the creativity of the science base."

Within Parliament itself, criticism was not confined to the opposition Labour Party — which said that it remained committed to an independent OST — but also came from the Conservative backbenches. In particular, Robert Jackson, who had been a junior minister in the Cabinet Office during the preparation of the government's white paper on science in 1993, wrote in an article in the *Financial Times* that the move represented "an alarming triumph of short-termism".

Taylor vigorously disputes such criticism, emphasizing the importance of assuring that "there is an interaction between what is happening in industry and what is happening in science", and thus the advantage to science — with its mission to improve the performance of the national economy and raise the quality of life — in being placed within the DTI.

Taylor was parliamentary private secretary to William Waldegrave, the cabinet minister responsible for science from 1992

to 1994. He says that part of his role will be to convince industry of the importance of long-term, 'blue-sky' research. "This is the most exciting portfolio in government, and the one that has the greatest opportunities," he says.

In response to concern expressed by, among others, Sir Arnold Wolfendale, former astronomer royal and president of the Institute of Physics, over the future of the government's support for the public understanding of science — an increasingly significant role of the OST — Taylor says that it "fits in beautifully" with his current efforts to promote awareness of the importance of industrial innovation, for example among school science teachers.

Taylor's appointment has come as some relief to the scientific community. He was previously under-secretary of state for trade and technology, a position that included responsibility for DTI's involvement in topics such as information technology and space, and is widely seen as both conscientious and approachable.

"We certainly welcome Taylor as someone who looks as if he will take a serious interest in his responsibilities," says John Mulvey, secretary of SBS. Space scientists, responsibility for whose field had previously been split between the DTI and the research councils, have given the appointment a particular welcome.

But Mulvey and other critics also point out that, however great Taylor's enthusiasm, his powers as a junior minister — for example, in his ability to influence the research agendas of other government departments — are likely to be limited.

Furthermore, by allocating responsibility for science to what is widely seen as a "third rank" ministerial post, the government may, according to some observers, have made it difficult for the House of Commons Select Committee on Science and Technology to make the case for its continued existence.

Taylor himself says that he considers the committee — which could in principle disappear under a post-1992 rule that the responsibilities of such committees must mirror those of government departments, placing science under a broader trade and industry committee (see *Nature* 376, 103; 1995) — plays an important role in reviewing science related issues.

Sir Giles Shaw, Conservative MP for Pudsey and chairman of the committee, says he is optimistic that it will survive, partly because of its unique transdepartmental role, and partly because the long-term issues it tackles, such as human genetics (see page 202), are relatively non-political.

David Dickson

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Taylor: arrival received a cautious welcome.

Universal Pictorial

Japan tackles downturn in science funding

Tokyo. Japan's science and technology has reached a turning-point, according to the latest annual 'white paper' on research released this week by the Science and Technology Agency (STA).

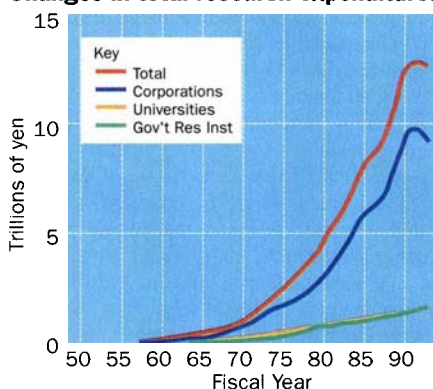
As a result of the country's prolonged recession, investment in research and development has begun to decline for the first time since the 1950s, while young people are showing less interest in science and technology.

After decades of almost exponential growth, for example, the document shows how Japan's expenditures on research fell for the first time in the fiscal year 1993, which ended in March 1994, due to reduced expenditure by the private sector.

A slight upturn in government expenditure as a result of numerous supplementary budgets was insufficient to offset the decline in spending, which has brought expenditure down from a peak of 2.78 per cent of gross national product (GNP) in 1990 to 2.66 per cent in 1993.

Adding to the gloomy picture is clear evidence that Japan's youth is showing less interest in science. Individuals in their forties and fifties have shown a steady increase in interest in science and technology over the past decade. In contrast, those in their twenties have shown a rapid decline in interest; now only just over 40 per cent

Changes in total research expenditures



say they are "very interested" or "somewhat interested" in news topics related to science and technology.

On top of this decline, the Kobe earthquake in January and the nerve gas attacks in Tokyo and Matsumoto have helped to undermine still further the faith of Japan's public in science and technology.

To overcome this situation, the STA document says that Japan must take steps to become a nation based on what it calls "creative" science and technology. Such exhortations have been repeatedly made in white papers over the past decade. But for the first time the agency lists some specific measures to revitalize Japan's government

research and development.

For example, the paper calls for more flexible employment of researchers to encourage mobility between institutions by, for example, offering higher pay to talented researchers and greater security for those on short-term contracts. Such suggestions may seem obvious to western researchers but are quite radical for Japan.

In line with these proposals, the agency echoes a popular government theme over the past few years by calling for the creation of centres of excellence that can attract talented researchers from Japan and other parts of the world.

It also calls for increased use of external evaluation of research institutes to assess the quality of research and administration. Since 1993, a handful of government institutes and university institutes — led by STA's Institute of Physical and Chemical Research (RIKEN) and Tokyo University's physics department — have brought in teams of external reviewers from Japan and overseas (see *Nature* 365, 97; 1995).

The 'white paper' says more research institutes should make use of such reviews. It also urges greater government investment in research and development. But given the poor state of Japan's economy, a dramatic change in this seems unlikely.

David Swinbanks

Vote on growth hormones in meat sparks row with FAO

London. The European Commission is deadlocked with the United Nation's main food agency over whether scientific evidence is sufficient to lift a ban on the importation and use of meat enhanced with growth-promoting hormones.

Earlier this month, the UN Food and Agriculture Organization (FAO), after a wide-ranging review, concluded that the European Union (EU) ban, which has been in force since 1989, lacked any scientific basis. It voted at a closed secret meeting on 6 July to approve the use of growth hormones in meat for domestic consumption as well as export among its member states.

But the European Commission — the body charged with overseeing EU legislation — remains unrepentant. Franz Fischler, the EU's agriculture commissioner, denounced the decision as unrepresentative, and confirmed that the ban on imports of hormone-enhanced meat would remain.

"The decision, which essentially was only supported by 20 per cent of the signatories of GATT, will have no bearing on the EU's policy on hormones," he says. The decision was adopted by 33 votes in favour, to 29 against, a majority of four, with seven abstentions and 25 spoiled ballot papers. Ninety-four nations out of 151 member

states were represented at the meeting. "If we are to adapt our policy, we will do so using our own decision-making process."

The decision was taken by the Codex Alimentarius Commission, a joint FAO/World Health Organization (WHO) body responsible for international food standards. The commission authorized the use in meat of three naturally occurring hormones, namely testosterone, progesterone and oestradiol, as well as two synthetic hormones, zeranol and trenbolone.

Growth-inducing hormones are used to produce leaner, more muscular meat. Hormones are administered before an animal goes to slaughter, and the maximum residue limits (MRL) are set below a concentration that induces hormonal effects in the animal.

The three naturally occurring hormones are not subject to safety levels as they are already present in the animal's body. Fritz Käferstein, head of food safety at WHO, says there is "no appreciable risk to humans" from consuming hormone-enhanced meat.

The Codex commission is not the only body opposed to the EU ban, as opposition also exists within the EU member states. The British government — which has consistently opposed the ban since its enforcement — issued a statement after the FAO vote

saying that it would take up the cause again when the European Commission holds a conference this autumn reviewing its approach to hormone use.

"The UK opposed the introduction of the initial ban on the use of hormonal growth promoters, on the grounds that the ban was not scientifically justified, and this remains the case," according to the statement issued by the Ministry of Agriculture, Fisheries and Food. "The UK welcomes the conference proposed by the commission to review the situation," the statement added. "But any decisions made as a result should be firmly based on science."

A ministry spokesman later explained that Britain felt that the EU ban was grounded in concern over competitiveness rather than scientific reasons. It was in force, he said, primarily to protect Europe's small meat-producers who, unable to afford hormone-enhancement facilities, might be wiped out by larger manufacturers from the United States if the ban was lifted.

Hormone-enhanced imports of US beef to the EU have been banned since 1989. The US government, which welcomed the Codex decision, had threatened to take the issue to the World Trade Organization if the ban is not revoked.

Ehsan Masood

Brent Spar or Broken Spur?

SIR — In the Commentary by E. Nisbet and C. M. R. Fowler on 29 June 1995 (ref. 1) and in your Opinion piece in the same issue², the significance of Brent Spar's cargo is measured by the yardstick of the discharge rate of metals by a natural system: the Broken Spur vent field on the Mid-Atlantic Ridge. You say that Broken Spur adds up to 5,000,000 tonnes of metals annually against which the hundreds of kilograms of metals on Brent Spar is insignificant. Simple comparison of numbers is misleading, because the forms in which metals are added to the environment influences their impact. But, in addition, the figures are wrong.

Nisbet and Fowler quoted their figures from preliminary estimates by R. W. N. and Murton³. These were based on metal data obtained in H. E.'s laboratory⁴ and were converted to rates by a procedure which, we now all agree, overestimated metal discharge rates by three to four orders of magnitude.

This is also apparent from other reasoning. The TAG vent field south of Broken Spur is the largest so far discovered and contains about four to five million tonnes of metals. This is similar to the annual rate you quote for Broken Spur, but we know that the TAG deposits have accumulated over 100,000 years⁵. Alternatively, estimates by us and others⁶ place the total annual global metal discharge rate from all vents at roughly 700,000–5,000,000 tonnes a year.

We believe in any case that this comparison is unhelpful. There was no intention to dump the Brent Spar on a vent field; mid-ocean ridge vent fields are sites of special scientific interest. Nisbet and Fowler are careful not to suggest this course, but the linking of Brent Spar with bacteria associated with vents, and the suggestion that mid-ocean ridges may be used as dump sites, could be considered to have this implication.

The total fluxes of all types related to processes within the mid-ocean ridge crest system are poorly known. The waste stored aboard Brent Spar could well act as an energy source for deep-sea bacteria, but may not necessarily benefit species already present; rather, they may be replaced by specialists better adapted to the changed conditions.

To be sure, the mass of the ocean is such

that it is likely that the global impact of one oil storage platform would be negligible. Of course, the total global mass of metals in the deep sea is large compared with what is in Brent Spar, but that is not the point. We do not argue for or against dumping in the deep sea, but rather emphasize that comparing figures such as these is oversimplistic. It does not constitute a measure of environmental impact. There is a danger in establishing a precedent in the absence of a proper evaluation, particularly as about a dozen other platforms are due for disposal before the end of the decade.

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SIR — Before accusing Greenpeace scientists of "shallowness"², perhaps you should have glanced at the documents produced by Shell in support of its original decision to dump the Brent Spar^{7,8}.

"Nobody pretends that the ocean floor is a garbage dump of infinite capacity", you state. Yet many of the unjustified assumptions on which Shell based their calculations amount to precisely that suggestion.

Shell reaches the conclusion, for example, that levels of heavy metals would be "negligible", largely by assuming that contaminants will be released slowly over a period of 1,000 years and distributed evenly within a 150-m radius of the source⁸. No data have been made available to support this assertion. The effects of finely dispersed hydrocarbons are described as "insignificant", based on extrapolation from unnamed fouling studies in the North Sea⁸. These studies are not identified in any way, nor are any data given. The toxicological responses of deep-water organisms are unknown and we are not aware of any toxicity tests that have been carried out on such organisms.

It is difficult to see how it could be claimed that the effect of dumping the Brent Spar would be "minimal or even beneficial", given present knowledge of deep-sea ecology and physical processes. It also ignores the political reality that the Brent Spar would have set a precedent for dumping other contaminated structures — each considered on an individual basis, but having

a cumulative effect on the environment.

Shell's dumping proposal is in no way justified by Nisbet and Fowler's hypothesis¹ that a "carefully chosen location on the Mid-Atlantic Ridge" might be an appropriate disposal site. Shell did not propose disposal at such a site, nor have any detailed studies been made of the effects of Brent Spar's contents at such a site — or indeed, any site. The precise nature of these contents is in any case poorly known and based on very limited measurements⁹. The knowledge that some organisms may benefit from heavy metals tells us nothing about how the ecosystem as a whole will respond to a cocktail of radioactive scale, heavy metals, hydrocarbons and trace quantities of PCBs.

Greenpeace is well aware of the problems of contaminated aquifers and has never proposed dumping in a landfill site as a suitable solution. Engineering firms are queuing up to show that they can dismantle the Brent Spar safely and effectively¹⁰, as has already been done with hundreds of oil installations in the Gulf of Mexico and nine in the North Sea itself¹¹. The pollutants can then be properly dealt with onshore. Treatment of oily wastes and sludge and removal of scale are operations which the oil industry already routinely carries out in land-based facilities.

Finally, we have never ignored the issue of overfishing — the MV *Greenpeace* is even now out seeking and cutting illegal driftnets. Backed, of course, by sound scientific arguments.

Helen Wallace

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SIR — The surrender of the Shell Oil Company to German public opinion over its oil rig is an example of democracy gone wrong and a disaster for future generations. As a result of the misguided lobbying of emotional environmentalists such as Greenpeace, the use of the oceans as a waste depository has been denied to applied scientists competent to take decisions.

Dumping waste in the shallow waters of the North Sea is obviously wrong, but dumping in the Atlantic Ocean several miles down is another matter. Consideration should also be given to dumping vitrified high-level radioactive waste, or even plutonium, sealed in long-life containers in a subduction zone where it would be carried into the mantle in the next 500 years. The missile tubes of nuclear submarines could be fitted with bottom doors to drop such containers to the seabed. The submarines' navigational equipment could locate the drop zone with great accuracy. The Tonga Trench in the Pacific is more than 6 km deep and hundreds of miles from large human populations.

W. M. Colles

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Genetics helping molecular dynamics

Analogy with natural evolution is the guiding principle of an essay in molecular dynamics that successfully predicts the structure of C_{60} , or buckminsterfullerene.

THE growing company of researchers in the physical sciences with an interest in solving problems in biology has frequently been applauded for its daring and ingenuity, as in the modelling of the cell cycle, the exploration of the dynamics of the self-assembly of molecular systems (the spontaneous construction of microtubules from tubulin, for example) and the specification of neural networks as analogues of how circuitry in the brain may work. Less attention has been paid to the occasions when biology has something substantial to offer physics.

Of course, nanotechnologists have already been stimulated to interesting rumination and experiment by the ubiquity of self-assembly in biological systems, at synapses for example. But here is something more tangible — the use of evolutionary principles to avoid the worst pitfalls in the technique of molecular dynamics, the now familiar way of telling the equilibrium configuration of a system of atoms by choosing that among all possible configurations whose energy is the least.

The obvious difficulty is that the number of possible configurations increases with the number of atoms in the system too quickly for the comfort of any finite computer; for a system of N atoms, all distinguishable, the number of ways in which they can be rearranged among each other will be of the order of $N!$. The more subtle, but also well known, difficulty is that many physical systems are distinguished by a large number of local energy minima in configuration-space. Computer programs designed to find the global energy minimum by means of small variations of the parameters of the atomic system will then be at a loss to tell whether they have found the true global minimum or some other.

The standard technique for escaping this pitfall, called 'simulated annealing', is figuratively to add a finite amount of kinetic energy to the system and then to take it away again in small decrements. If a false minimum is separated from the global minimum by a modest potential barrier, the computer model of the system may find its way to the true minimum, and its equilibrium configuration will be determined. There are two snags. Simulated annealing is a great thief of computer time, and there is no objective way of testing whether the energy minimum eventually found is truly the global minimum.

This is where biology may help. Molecular dynamics seeks to find an optimum configuration, one whose free energy is the

least. Evolution by natural selection is also a means by which the consequences of the genetic attributes of a species (its phenotype) are optimized to the local environment. But natural selection has the advantage over simulated annealing that sexual reproduction is forever offering novel genetic arrangements on which selection can be allowed to work. So why not use the analogy of evolution to find energetic optima for an atomic system?

The idea is far from new, but D. M. Deaven and K. M. Ho, from the US Department of Energy's Ames Laboratory in Iowa, have now applied it to the 'prediction' of the well known truncated icosahedral structure of molecular C_{60} , otherwise buckminsterfullerene (*Phys. Rev. Lett.* **75**, 288–291; 1995). They say attempts to solve this structure by conventional molecular dynamics have been unsuccessful.

There is more to the procedure than putting 60 carbon atoms in a box, specifying a field of force between them and then seeing what configuration emerges in the course of time. Because the procedure is meant to simulate evolution, there has to be a population of trial molecules at each stage of the enterprise. And, crucially, pairs of molecules must be able to 'mate' with each other to produce a 'child'.

How? Deaven and Ho follow a simple rule: they draw the same randomly chosen imaginary plane through the centre of gravity of the two partners to the mating, stick the top half of one and the bottom half of the other together to form the child of the mating and then put the child through conventional molecular dynamics. Because the two halves may not have 30 carbon atoms each, the program will displace the mid-planes by equal but opposite distances until the child contains just 60 atoms.

Various refinements make the program manageable. If all possible matings were allowed, the number of the candidate molecules in the 'population' would be squared at each new generation, but good sense demands that it should be constant. That is done by choosing the partners for each mating by a numerical rule embodying a bias in favour of potential 'parent' molecules whose energy is towards the low end of the whole range. (Deaven and Ho use as a weighting rule a simple Boltzmann negative exponential in which the numerator of the exponent is the energy of the putative parent and the denominator is the total range of the energy of the parents.)

That rule is the program's analogue of

natural selection; low-energy configurations will contribute preferentially to the next generation, but the chance that high-energy configurations will have an influence is not excluded. Evidently, that is how the algorithm concentrates on the evolution of the putative molecular structure towards a particular minimum while taking account of regions of configuration space far away from what may be a possibly spurious local minimum.

The outcome is pleasing. Starting with a variety of initial configurations, the authors reach the buckyball configuration after 5,500 mating generations. Interestingly, the algorithm appears to hang up on various incorrect intermediate structures; in one illustration, a form of C_{60} in which two pentagons are adjacent to each other was converted into true buckminsterfullerene only after 2,000 mating generations. The calculated binding energy is 9.4 eV per atom, not far from reality.

One snag with the approach is that, while C_{60} apparently gives an unambiguous result, superficially simpler molecules such as C_{20} do not; the simple evolutionary algorithm yields a structure in which 20 carbon atoms form a simple circular ring. So the authors describe a process called 'mutation' in which the positions of the atoms of an evolving cluster are periodically altered, in a predetermined fraction of an offspring generation, according to one of other of two recipes. (Either all atoms are sent on a randomly specified brownian random walk, or some configurations are moved against the force gradient into a neighbouring watershed of potential.)

That way, the authors can be sure of including the influence of radically different structural classes, which will come to the fore only if they are energetically favourable. For C_{20} , the authors thus recapture the cap-like structure formed when six benzene rings are cemented together around a pentagon to form one end of a buckyball.

It will be interesting to see what can be made of more complicated molecules. The technique seems ideally suited to that old biological question of protein folding. The mating procedure would seem well designed to make the best of circumstances in which a protein evolving towards its native condition is represented by well-folded half-chains in separate members of some generation. But even as things are, computers are probably not powerful enough to say very much. **John Maddox**

Upside-down ideas vindicated

Brigid L. M. Hogan

IN 1822 the French zoologist Geoffroy St-Hilaire proposed that vertebrate and arthropod embryos have a common body plan, but an inverted dorsal-ventral axis¹. In other words, *Drosophila* larvae resemble upside-down tadpoles, and Kafka's protagonist, Gregor Samsa, crawled around on his former back rather than his belly after metamorphosing into an insect. Over the years, Geoffroy St-Hilaire's thesis of dorsal-ventral inversion has fallen into disrepute, but recent progress in our understanding of embryonic pattern formation has revived the debate², and now support comes from compelling functional data presented by Holley *et al.* on page 249 of this issue³.

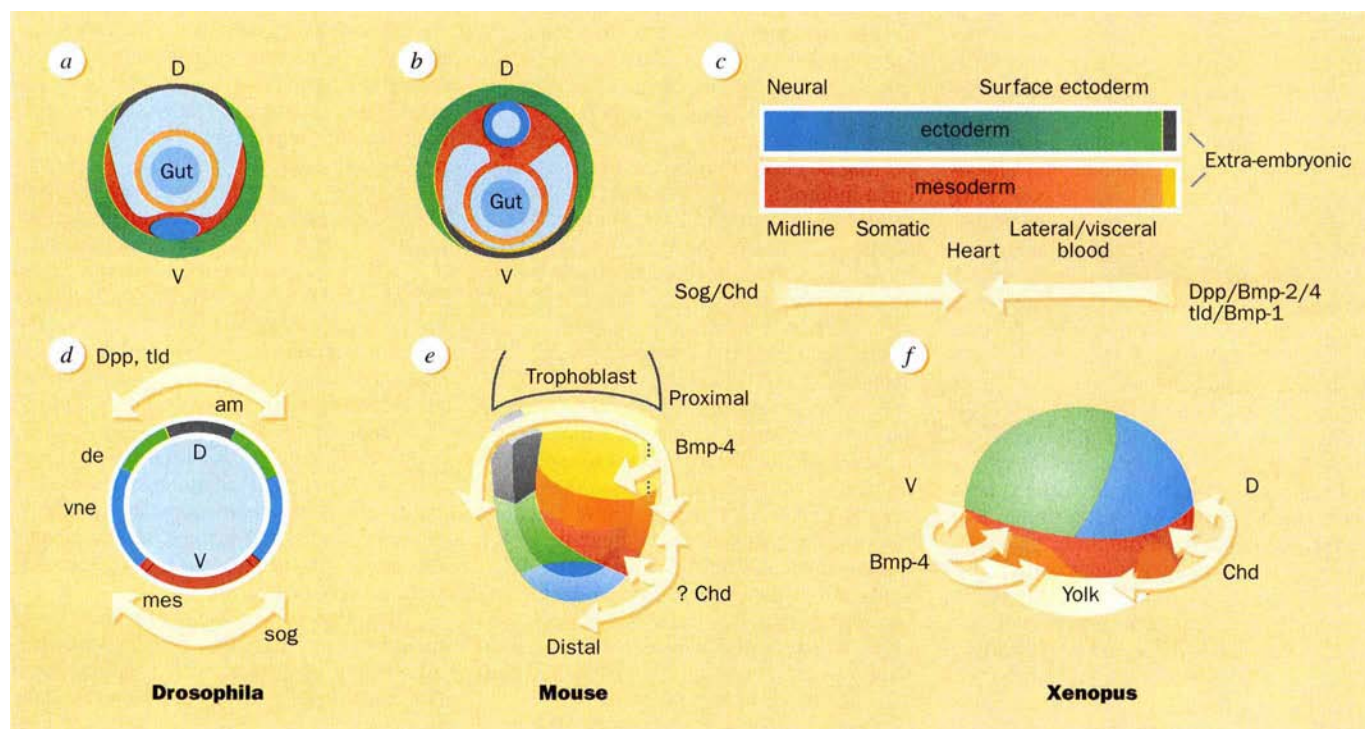
These authors find that the products of the *Drosophila* gene *decapentaplegic* (*dpp*) and the *Xenopus* gene *bone morphogenetic protein-4* (*bmp-4*) can promote

either dorsal or ventral patterning, depending on whether an insect or vertebrate embryo is tested. In either case, the inducing activity is antagonized by the products of the *Drosophila* *short gastrulation* (*sog*) or *Xenopus* *chordin* (*chd*) genes. The results suggest that the two classes of signalling molecules represent counteracting systems that control dorsal-ventral patterning and might have been established in a primitive ancestor before the divergence of the arthropods and vertebrates.

What kind of signalling molecules do these genes encode and whereabouts are they expressed in the early embryo? *Dpp* and *Bmp-4* are both secreted proteins of the TGF- β growth-factor superfamily. *Bmp-4* is closely related to another vertebrate protein, *Bmp-2*, and both are about 75 per cent identical to *Dpp*. In *Drosophila*-

la, *dpp* messenger RNA is expressed in the dorsal half of the blastoderm embryo; and in null mutants, tissues derived from this region, including the amnioserosa and dorsal epidermis, are ventralized, so that the denticle bands normally confined to the ventral epidermis encircle the larva⁴. In the *Xenopus* blastula, *bmp-4* RNA is specifically absent from the region around the dorsal lip⁵. In the mouse embryo, *bmp-4* transcripts are highest in the posterior primitive streak and ventral and extra-embryonic mesoderm⁶, whereas *bmp-2* transcripts are abundant in the developing amnion (H. Zhang and A. Bradley, personal communication).

Null mutations have recently been generated in both the mouse *bmp-4* and *bmp-2* genes: most *bmp-4* mutants arrest before gastrulation, but those that develop further are deficient in posterior and extra-embryonic mesoderm (unpublished results from my laboratory); the primary defect in *bmp-2* null mutants is failure of early amnion formation (H. Zhang and A. Bradley, unpublished results).



Schematic representation of the body plan and fate maps of *Drosophila* (insect) and vertebrate embryos and the proposed conserved dorsal-ventral (D-V) patterning systems. *a* and *b*, Cross-section of an idealized insect or arthropod (*a*) and vertebrate (*b*) showing the opposite D-V orientation of the CNS (nerve cord, neural tube: blue), surface epidermis (green), extra-embryonic ectoderm (amnioserosa, amnion: grey), midline mesoderm (somatic muscle, notochord: red), lateral mesoderm (visceral muscle, splanchnopleure: orange) and extra-embryonic mesoderm (amnion, yolk sac: yellow). *c*, Proposed morphogenetic systems affecting the patterning of ectoderm and mesoderm. The factors are shown as acting on the two germ layers independently, but they may interact. In *Xenopus* and mouse there is evidence for other dorsalizing factors such as *noggin* and *nodal* (also of the TGF- β superfamily). *Drosophila* *tolloid* (*tld*) encodes a putative metalloproteinase which enhances the *dpp* signalling pathway and is related to vertebrate *bmp-1*. *d-f*, Schematic fate maps of early embryos. *d*, Fate map of *Drosophila* blastoderm embryo showing

relative positions of the amnioserosa (am, grey) dorsal epidermis (de, green), ventral neurogenic ectoderm which gives rise to both neural tissue and ventral epidermis (vne, blue), and mesoderm (mes, red). *e*, Fate map of the early gastrula mouse embryo¹⁰. The epiblast is cut down the proximal-distal axis relative to the extra-embryonic trophoblast. Colours show the relative domains of the prospective amnion (grey), surface ectoderm (green), CNS (blue), extra-embryonic mesoderm (yellow), lateral mesoderm (light orange), somitic mesoderm (light red), heart (dark orange) and notochord and prechordal mesoderm (deep red). The putative *bmp-4*- and *chd*-based morphogenetic systems are centred around the proximal and distal ends of the primitive streak (dotted line) respectively. The distal end of the streak corresponds to the future node. *f*, Fate map of the late-blastula-stage *Xenopus* embryo¹⁰. Colours correspond to the same prospective tissues as in mouse. The high point of dorsalizing activity corresponds to the future dorsal lip of the blastopore; the ventralizing activity is postulated to centre around the future ventral lip.

The *sog* and *chd* genes both encode large extracellular proteins with repeats of a conserved cysteine-rich domain⁷. In *Drosophila*, *sog* expression is the reciprocal of *dpp*, with transcripts localized to the ventrolateral regions of the embryo, and in null mutants there is a reduction in the amount of ventral neurogenic ectoderm and a narrowing of the denticle bands. Moreover, genetic evidence shows that Sog antagonizes the dorsalizing activity of Dpp⁸. One explanation for this is based on the fact that Dpp appears to be a true extracellular morphogen, with different concentrations determining different cell fates; by binding to Dpp, or to Dpp-receptor complexes, Sog would effectively reduce the amount of active Dpp to a lower threshold level. In the *Xenopus* embryo, *chd* is the reciprocal of *bmp-4*, with RNA initially localized to the dorsal lip of the blastopore and the midline mesoderm derived from it⁹. A mouse *chd*-related gene has been identified, but the mutant phenotype is not yet known.

Holley *et al.*³ microinject RNA into embryos to show that *dpp* and *bmp-4* are functionally interchangeable, and likewise *sog* and *chd*. Moreover, a factor that has dorsalizing activity in *Drosophila* has ventralizing activity in *Xenopus*, and vice versa. For example, injecting *sog* RNA into a vegetal blastomere of a *Xenopus* embryo ventralized by irradiation with ultraviolet light rescues a full dorsal axis. By contrast, injection of *dpp* RNA into animal blastomeres ventralizes the embryo, but dorsal development can be rescued by injecting *sog* into just one vegetal blastomere, showing that its effect is not cell-autonomous.

How do these results fit into a molecular model that would vindicate Geoffroy St-Hilaire's radical ideas? One approach is to compare the fate maps of *Drosophila*, *Xenopus* and mouse embryos (see figure), and to project onto them the two proposed morphogenetic systems. One, involving *sog/chd*, promotes the differentiation of neurectoderm and midline mesoderm and somatic muscle, and has highest activity in the region from which these tissues arise. The second, based on *dpp/bmp-4*, promotes the differentiation of epidermis and visceral mesoderm and

blood, and is likewise centred around the precursors of these tissues, as can be seen in the figure.

When the fate maps are compared in this way, it is striking that the spatial organization of the prospective central nervous system, epidermis and amnion, and somatic (midline), visceral and extra-embryonic mesoderm is the same in all three organisms¹⁰. Consequently, the polarity of the morphogenetic systems has also been conserved, but relative to each other, rather than to the final dorsal-ventral axis of the organism. This suggests that with the divergence of the insects/arthropods and vertebrates, the two systems became aligned with respect to the

STOCHASTIC RESONANCE

Neurons in parallel

Frank Moss and Xing Pei

THE property of certain nonlinear systems whereby a random process — the noise — can optimally enhance the detection of a weak signal is called stochastic resonance (SR). SR has been demonstrated in numerous physical and two biological experiments^{1,2} which, however, share a common feature: the noise and signal were applied to a single nonlinear element. Yet in biology, the parallel detection of signals by populations of sensory neurons is the rule rather than the exception. The exquisite sensitivity of many animals to weak external stimuli and their remarkable ability to distinguish weak coherent signals in the midst of large environmental noise provides a strong reason for looking at SR in parallel systems³. Moreover, contemporary engineering research in nonlinear signal processing is moving towards parallel processing in very-large-scale integrated circuits.

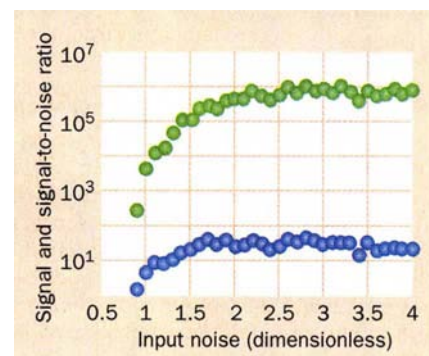
On page 236 of this issue, J. J. Collins *et al.* describe just such a theoretical and numerical study⁴. They consider a set of N model neurons, each with an internal noise, whose inputs are tied to the same weak (sub-threshold) signal source and whose outputs are summed. One immediately imagines a bundle of sensory neurons, for example, muscle spindle afferents. Each neuron fires spontaneously and incoherently, but all are subject to the same weak stimulus, and all converge to a summing centre. The signal processing power of such an array is immediately evident. The individual, incoherent noises increase only by a factor $\sqrt{N}$ in the average across the population, while the common stimulus is additively enhanced (by the factor N).

The reported results, apart from some technical details, demonstrate this as the standard behaviour. This much is not surprising. But an unexpected result is

dorsal-ventral axis in opposite ways. All the experimental data so far available are compatible with this model, but many gaps must be filled. It will be interesting, for example, to see the effect of disrupting the mouse *chd* gene, and to know whether the patterning function of other components of the *dpp* system, such as *tolloid* (*tld*) and *schnurri* (*shn*), have also been conserved during the evolution of the vertebrates. □

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that, in the limit of large N , the dependence of the processed signal's coherence on the noise intensity disappears. Thus there is no optimal noise intensity for large arrays, only a necessary minimal intensity. This observation neatly addresses the following question. If arrays of neurons make use of SR, then why do we observe a large range of spontaneous noise intensities across typical populations? The present finding of noise independence means



Signal strength (green circles) and signal-to-noise ratio (blue circles) versus external noise intensity from a simulation of 80 parallel ion channels. The dependence on noise intensity nearly disappears above a certain minimum, as found by Collins *et al.*⁴.

that processing in such arrays is already optimal, and no physiological mechanism for adjusting the internal noise intensity (to some optimal value) need be sought.

But even within biology, the present model may be more general. A set of ion channels in a cell membrane, exposed to a common extracellular signal, are connected in parallel in just the way envisaged here. Moreover, each channel fluctuates randomly between open and closed states in response to local thermal fluctuations (which are the ultimate source of the

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noise). In a remarkable experiment using a parallel array of up to 100 ion channels, formed from voltage-dependent alamethicin peptides in a lipid bilayer, Bezrukov and Vodyanov⁵ have observed both signal enhancement and SR — the first such demonstration at the molecular level. Inspired by this experiment, and to illustrate the versatility of the model developed by Collins *et al.*, we have replaced the Fitzhugh–Nagumo neurons that they used with simple open–close threshold elements (described in ref. 3). When the internal noise crosses the threshold, the channel opens for a random time. All elements were exposed to the same weak, periodic signal plus a common external noise. The results, which accurately mimic those of the ion-channel experiment, are shown in the figure: as Collins *et al.* found, the dependence on noise intensity nearly disappears above a certain minimum.

The individual elements in the model by Collins *et al.* are uncoupled, thus representing perhaps the simplest possible multi-element SR system. By contrast, the emphasis of recent numerical and theoretical studies has been on systems with coupling, either global or diffusive. For global coupling, Jung *et al.*⁶, and Inchiosa and Bulsara⁷, have shown that there exists not only an optimal noise intensity but also an optimal coupling strength, whereas diffusive coupling leads to pattern formation and spatio-temporal SR^{8–10}. In two-dimensional arrays, spatio-temporal SR manifests itself as the noise-induced optimal formation of coherent structures, chiefly spiral and target waves^{9,11}.

The next challenges for experimentalists are searches for SR in parallel sets of nerve fibres; coupling effects in synaptically interconnected networks; and spatio-temporal SR, perhaps in arrays of ion channels where second messengers provide diffusive coupling or in interconnected networks. All of these phenomena are well established by physical theories and numerical simulations, but have yet to be found in real life. □

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Cooling the tropics

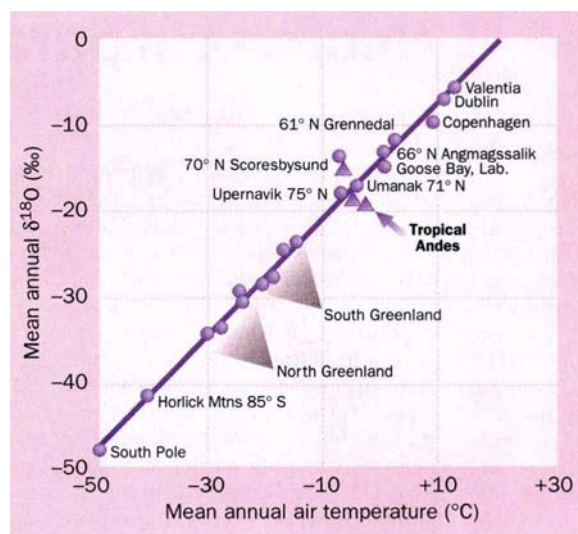
W. S. Broecker

UNTIL recently our reading of the palaeoclimate record favoured the viewpoint that during glacial time the tropics were spared the cooling which plagued the Earth's high-latitude regions. Evidence from marine sediments, based on the species makeup of the shells of planktonic organisms, the oxygen isotope composition of foraminifera calcite, and the alkenone composition of organic matter, appeared to limit the cooling of the tropical ocean to no more than 2 °C. Seemingly contradictory evidence based

this month in *Science*², new fuel has been thrown on the already hot fire of this debate. Lonnie Thompson and his colleagues at Ohio State University document an 8‰ glacial to Holocene change in the oxygen isotope composition of glacial ice from 6 km elevation in the tropical Andes. Not only does this result fortify the snowline-based conclusion that, during glacial time, high mountain temperatures were 5° or so colder than today, but also sows the seeds for an explanation as to how the Earth succeeded in making itself so cold during glacial periods. The combined snowline and oxygen isotope results point to lower inventories of atmospheric water vapour — so much lower that the resulting reduction in the Earth's greenhouse blanket would have produced a global cooling of several degrees.

But before discussing its scientific aspects, we should note that this story has another important dimension. It reveals how the tenacity of a lone scientist moving against the grain of conventional wisdom can alter the course of thinking. While most scientists in his field were obsessed by the record kept in ice cores from Greenland and Antarctica, Lonnie Thompson pushed to extend these studies to small glaciers capping the Earth's highest mountains. For years, he fought not only the cold condition of his field sites but also the lukewarm reception by many of those in our field (including me). In 1993, with the help of a small army of strong bodies recruited in Peru, he succeeded in hauling 6 tons of equipment to a col located at 6,050 metres elevation on Huascarán in the Andes (9° S). Power derived from solar panels allowed him to drill two 160-metre-long cores to bedrock. Ten tons of equipment and ice had to be lugged back down. This accomplishment alone places Lonnie Thompson in the ranks of our great explorers.

But Lonnie had an ace up his sleeve. He was convinced that the ice at the base of his cores would prove to be glacial in age. This confidence was based on the fact that at this elevation the glacier would remain firmly frozen to the underlying rock rather



Plot showing the relationship between the mean annual oxygen isotope composition of precipitation and the mean annual temperature for high-latitude regions³. The three triangles are for Holocene-age snow in the tropical high Andes⁵. They cluster around the high-latitude trend line. As the slope of the trend line for high latitudes is consistent with a simple Rayleigh condensation, the concordance of the Andean snow compositions supports the concept that their isotope composition provides a measure of the fraction of remaining water vapour. ($\delta^{18}\text{O} = (^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}} - 1$, using the SMOW standard).

on a 900-metre lowering of tropical snowlines during glacial time tended to be passed off as the result of steepened lapse rates or enhanced snowfall. But palaeotemperatures based on two new proxies have forced this long-standing conclusion to be re-evaluated. Strontium to calcium ratios in corals¹ and noble gas concentrations in ground waters (M. Stute, personal communication) suggest that tropical temperatures dropped by 5 to 6 °C during peak glacial time. Further, at the Baltimore meeting of the American Geophysical Union in May, new results by Bill Curry of the Woods Hole Oceanographic Institute drove a wedge into the seemingly invincible oxygen isotope constraint.

Finally, in an article published earlier

than flowing away. Not only did his predictions prove correct, but the glacial ice proved to have two amazing properties. First, as mentioned, its ^{18}O to ^{16}O ratio was 8‰ lower than for the overlying Holocene ice. Second, its dust content was two orders of magnitude higher than that for Holocene ice.

Whereas it is not difficult to picture what the incredible dustiness of Huascarán glacial ice tells us about the climate in upwind Amazonia, the significance of the ^{18}O shift requires discussion. At high latitudes, the oxygen isotope composition of precipitation closely follows expectation based on a simple Rayleigh condensation model. Because the vapour pressure of H_2^{18}O is one per cent lower than that for H_2^{16}O , as an air mass cools, its remaining vapour is progressively depleted in the heavy isotope. So also then is the precipitation formed in this air mass. This creates a tie between air temperature and the oxygen isotope composition of the precipitation. In the Earth's cold regions,

the mean annual temperature and $\delta^{18}\text{O}$ of snow are highly correlated (as can be seen from the figure), and the slope of 0.7‰ per $^{\circ}\text{C}$ is consistent with expectation³. A drop of 8‰ in $\delta^{18}\text{O}$ corresponds to a cooling of 11 $^{\circ}\text{C}$. As the $\delta^{18}\text{O}$ for any given snow depends on the difference in temperature between sea surface and the precipitation-generating cloud, if sea surface temperatures were lower during glacial time, the mountain top cooling required for an 8‰ shift would have to have been still larger.

Of course, as the factors influencing the oxygen isotope composition of snow falling on high tropical mountains are probably more complex than those for Greenland and Antarctica, the Rayleigh model may not be applicable to Huascarán. But I find it difficult to come up with any explanation accounting for both the snow-line lowering and the oxygen isotope shift which does not call for a substantial reduction in both tropical temperatures and water vapour inventories.

For those of us in search of a mechanism capable of propagating the effects of reorganization of the oceans' thermohaline circulation across the globe⁴, Lonnie Thompson's discovery points us to the water vapour pumping capacity of tropical convection systems. His findings should also send a strong warning to those who choose to discount the potential of the ongoing greenhouse buildup. The palaeoclimate record shouts out to us that, far from being self-stabilizing, the Earth's climate system is an ornery beast which overreacts even to small nudges. □

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QUANTUM PHYSICS

Gregarious atoms

Christopher J. Foot and Andrew M. Steane

ONLY last month, one of us remarked in these pages¹ that work by Petrich and co-workers² was bringing the long-sought Bose–Einstein condensation in a gas tantalizingly close. Bose–Einstein condensation (BEC) can be regarded as a quantum effect which occurs at low energies when the wavefunctions of atoms overlap so that the individual particles become indistinguishable. The whole sample must then be treated as a single quantum object with properties such as coherence. Now, the same group has observed such condensa-

tion in a gas of rubidium-87 atoms³.

The team of Anderson, Ensher, Matthews, Wieman and Cornell, working at the Joint Institute for Laboratory Astrophysics in Boulder, Colorado, confined the ^{87}Rb atoms in a magnetic trap and cooled them to the lowest kinetic temperature yet reached — only a few nanokelvins — so that thousands of atoms collected in the same quantum state at the bottom of the magnetic trapping potential. This was possible because ^{87}Rb atoms are bosons — particles whose total angular momentum

is an integer multiple of $\hbar/2\pi$. Such particles behave in the way first described by Bose and Einstein. Bosons are gregarious—more precisely, the probability that a process will result in two bosons having exactly the same quantum state is proportional to one plus the number of bosons already in that state. A short reflection will show that this leads to the possibility of an avalanche process, like a run on the stock market, in which as more bosons (or currency notes) enter a given state, other bosons join them more and more quickly, until the supply of bosons (or money) runs out. Just such a process was observed by Anderson and colleagues. By contrast, members of the other fundamental class of particles, the fermions (Fermi–Dirac particles) keep

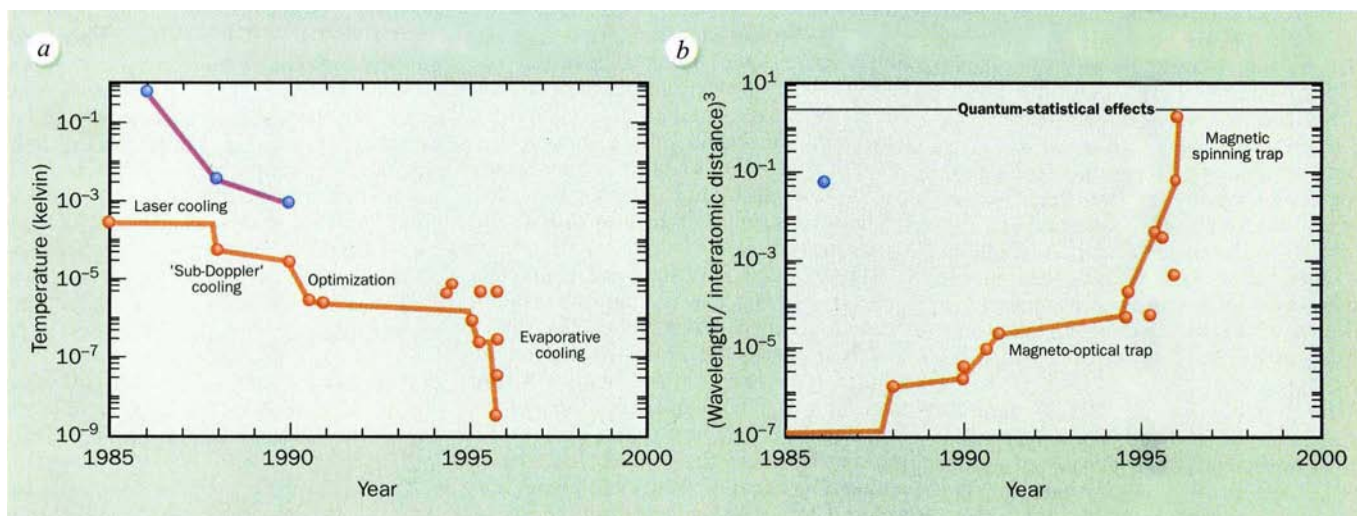


FIG. 1 *a*, Progress in cooling of atoms by experimental groups around the world over the past decade. The major reductions in temperature are labelled with the cooling techniques used to achieve them. *b*, A similar plot for phase-space density. The horizontal line at approx-

imately 2.6 marks the point at which quantum-statistical effects, such as BEC, become significant. The new experiment³ has achieved phase-space densities of over 100. Blue points, hydrogen; red points, alkali metals (Na, Cs and Rb).

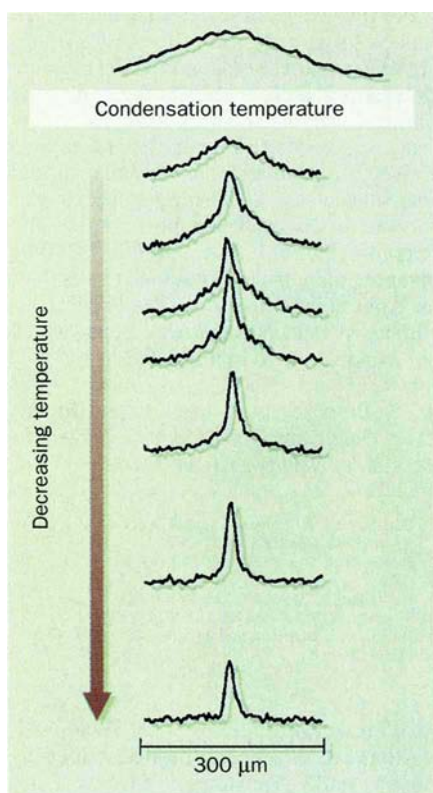


FIG. 2 Velocity distribution of the trapped atomic cloud in the experiment of Anderson *et al.*³ at various temperatures. Above the condensation temperature, the velocity distribution is smooth and fairly uniform. As the temperature decreases below the condensation point, a distinct bump corresponding to the condensed atoms appears, and grows until it contains a large fraction of the trapped atoms. Because the condensed atoms are in the lowest energy state of the trap potential, they occupy only a narrow range of velocities.

apart. It is impossible to have more than one fermion in a given quantum state. This exclusion principle governs the electronic structure of atoms, because electrons are fermions.

A remarkable feature of the work at JILA is that it did not rely on hugely expensive items of equipment but rather on the refinement of known techniques with one very clever innovation. The group developed a TOP-trap, standing for time-orbiting potential. As described in the earlier News and Views article¹, the atoms see a time-averaged magnetic potential which allows cooling to temperatures over three orders of magnitude lower than that of the laser-cooled atoms loaded at the start of each experimental run.

The previous work at JILA² demonstrated that the TOP-trap could be loaded with a sufficiently high density of atoms that the interatomic collisions quickly established thermal equilibrium in the gas. This allowed cooling by forced evaporation: atoms of above average energy were removed from the trap so that after re-thermalization the average

energy was lowered. That this really is a breakthrough can be seen by looking at Fig. 1. Laser-cooling itself dramatically lowered the temperatures which could be obtained, but only gradual improvements have been made in the past five years.

Also shown is the phase-space density which is a parameter roughly equal to the number of particles per state of the system; BEC occurs when it is greater than 2.6. One can see that the work on spin-polarized hydrogen has been close to BEC for some while. These extremely demanding experiments were thought by many to be the most likely route to succeed. Indeed, the evaporative technique was first developed to cool magnetically trapped atomic hydrogen, and such work helped to stimulate the current great interest in BEC. Simple laser-cooling has been carried out for atomic hydrogen, but a source of Lyman- α radiation is necessary. Heavy alkalis, in contrast, need near-infrared light which can nowadays be obtained easily from diode lasers.

The new experiment also used the laser light to probe the distribution of atoms in the trap. The first sure indication of something interesting going on was that the absorption of the cloud rapidly increased as the temperature reached a few nanokelvins, corresponding to densities rising to around 10^{15} cm^{-3} . More specific evidence for condensation was obtained by taking a 'flash photograph' of the cloud of atoms. When a laser beam was sent through the cloud on to a CCD camera, the shadow of the cloud was detectable. The light was flashed on for a short time (20 μs) to get a true picture of where the cloud was before the atoms were pushed away by the radiation force.

The atomic distribution observed in this way (see Fig. 2) gives direct evidence of BEC where one can actually 'see' the condensate as a peak rising up out of the surrounding cloud as the temperature decreases. This is a beautiful result. One of the big surprises was that so many atoms condensed — the loss rates were uncertain before this experiment. Fortunately the condensate (of several thousand atoms) lasts for around 18 seconds, which is ample for experiments.

The appearance of the peak is in itself quite convincing, but the authors present even stronger evidence. When the trap is at full strength, the atoms in the lowest energy state are confined within a few micrometres. This is difficult to image, so the distributions shown in Fig. 2 were measured after the cloud had been allowed to expand. When the cloud was allowed to expand still further, the condensate fraction expanded more quickly in the axial direction than in the radial direction whereas the surrounding atoms expanded uniformly in both directions.

This behaviour is just what is expected for a Bose-Einstein condensate. Uncondensed atoms in the trap are in thermal equilibrium — this is necessary for evaporative cooling as explained above — and hence the components of velocity in all directions are the Maxwell-Boltzmann distributions well known in classical gases. But in the condensate the classical description is no longer adequate. Quantum mechanically the shape of the trap is closely modelled by a harmonic oscillator potential, which has a parabolic cross-section. This potential is narrower, and thus more tightly confining, along the axial direction than radially (the ratio of the oscillation frequencies in these two directions is $\sqrt{8}$ for the TOP-trap).

Thus the wavefunction representing the condensed atoms is oblate (squashed along the axial direction). This leads, through the Heisenberg uncertainty principle, to a difference in the momentum spread along the two directions. As the condensate is initially smaller along the axial direction it will have a larger spread in momentum along this direction so that the cloud becomes elongated (prolate) when it has expanded sufficiently for the initial size to be negligible. This elongation of the cloud was indeed observed. Furthermore, the weak repulsive force which exists between the atoms is increased by the presence of condensation and also contributes to the expansion. However, the interactions in this atomic gas are still much weaker than in other systems such as superfluid helium, and the sample remains gaseous throughout.

One of the advantages of this system is that the interactions can be controlled by varying the density and, theoretically, can be tuned by an applied magnetic field. (There have been reports of BEC in an excitonic gas; this, however, is a very different system, because the excitons are short-lived and are confined to the solid object in which they exist.) The new method could be extended to other atoms so that coherent samples of different species can be made. Such samples will be sources of coherent matter-waves and have many potential applications. One of the favourite possibilities is to make an atom laser, a system for matter-waves which is analogous to the laser for light. A remote possibility? Perhaps, but then BEC seemed out of our reach only a few months ago. $\square$

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Polar explorations

David D. Hackney

IN 1993, determination of the structure of myosin subfragment-1 and its complex with F-actin^{1,2} provided a fruitful basis for investigations into how force is generated at the molecular level. Three papers in this issue³⁻⁵ (pages 271, 274 and 277) now report the first three-dimensional reconstructions of the monomeric motor domains of the structurally similar kinesin and *ncd* proteins bound to the tubulin subunits of a microtubule. Members of the kinesin superfamily of motors are responsible for movement of a range of different cargoes along microtubules, and they are involved in such diverse processes as vesicular transport and chromosome movement (see ref. 6 for review).

The kinesin superfamily is defined by a conserved motor domain or head of relative molecular mass about 38,000. The head domain possesses a microtubule-stimulated ATPase activity and it is sufficient on its own for generation of movement when linked to a nonspecific tail. Kinesin itself has the motor domain at the amino terminus, but some other superfamily members such as *ncd* (which stands for non-claret disjunctional) have the motor domain at the carboxy terminus. Intriguingly, all amino-terminal motors that have been tested move towards the 'plus' end of the microtubule, which is where the tubulin subunits are being added more rapidly, whereas carboxy-terminal motors move towards the 'minus' end. In both cases an extended region of coiled coil produces dimerization that may be important for generation of processive movement^{7,8}. But more detailed structural information is necessary to elucidate the mechanism of motility.

Kinesin motor domains decorate unfolded microtubule sheets with a shallow helical pitch and 8-nm periodicity that corresponds to the spacing of tubulin $\alpha\beta$ heterodimers along the protofilament in a B-lattice⁹. The B-lattice with the usual 13 protofilaments generates a discontinuity where a row of β -subunits abuts a row of α -subunits (see figure). This seam has been directly observed¹⁰. Microtubules with either 10 or 16 protofilaments should not contain a seam¹¹, and helical averaging can be applied during image recon-

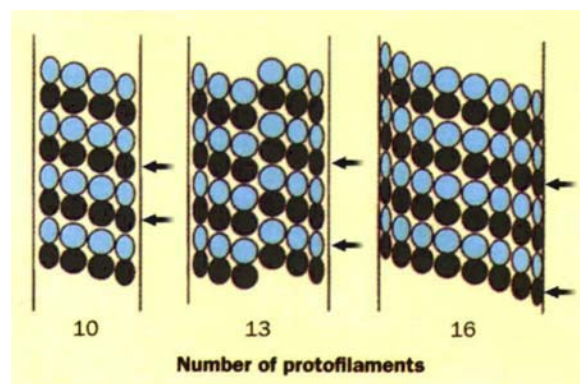
struction. Kikkawa *et al.*⁴ used microtubules prepared by polymerization of purified tubulin and then laboriously examined many images to find the few that had only 10 protofilaments. Hirose *et al.*⁵ used microtubules with 16 protofilaments that occur naturally in the sperm axonemes of some insect species. Hoenger *et al.*³ used flat sheets of unfolded microtubules and obtained three-dimensional information on *ncd* by examining tilted specimens.

The three structures agree in a number of ways. In each case the head domain is not highly asymmetric; is centred above one tubulin subunit; makes less extensive contact with a second tubulin subunit along the protofilament; and makes little lateral contact with the neighbouring protofilaments. Binding of the motor domains also induces large conformational changes in the microtubule. Crosslinking experiments indicate that the principal contact is with a β -tubulin subunit^{9,10}, but crosslinking to α -tubulin has also been observed¹².

Both Hoenger *et al.*³ (*ncd*) and Kikkawa *et al.*⁴ (kinesin) observe the head to tilt to the right when viewed from the outside of the microtubule with the plus end at the top. The secondary contact is with the tubulin subunit on the plus side of the primary contact. The orientation of the kinesin head in the reconstruction by Hirose *et al.*⁵ is reversed, however, with a tilt to the left and the secondary contact located towards the minus end. Although the direction of tilt for *ncd* and kinesin need not be the same, both Kikkawa *et al.*⁴ and Hirose *et al.*⁵ used essentially identical kinesin constructs in the presence of an unhydro-

lysable ATP analogue, AMP-PNP, and still observed different directions of tilt. Either the resolution of these reconstructions is insufficient for distinguishing between these subtle differences in orientation and shape, or the polarity has been misassigned in at least one structure. The assignment of polarity is also currently a matter of controversy in regard to the identity of the terminal subunit at the plus end as either α (ref. 13) or β (ref. 14).

The polarity is critical because of its importance for generation of directed movement. If *ncd* and kinesin are tilted in opposite directions, then this immediately



A novel aspect of the three papers³⁻⁵ discussed here is the use of microtubules with abnormal numbers of protofilaments but true helical symmetry, as depicted schematically above. A tubulin $\alpha\beta$ heterodimer is indicated by a grey and blue circle in contact along the protofilament. For the plus end at the top, and the polarity of Hirose *et al.*¹⁴, the blue circles are β -subunits. For the polarity of Song and Mandelkow¹³, they are α -subunits. The arrows indicate the repeat of a continuous row of subunits. Microtubules with 10 and 16 protofilaments are the largest and smallest arrangements that form stable 1 and 2 start helices with a tubulin $\alpha\beta$ heterodimer as the repeating unit¹¹. The 10-protofilament microtubule is designated as a 1 start helix because it consists of a single continuous strand of $\alpha\beta$ dimers. The rise per turn of 8 nm (between arrows) equals the spacing of dimers along the protofilament. The 2 start microtubule with 16 protofilaments has two continuous strands of dimers and each strand completes a full turn with a rise of 16 nm. The common 13-protofilament microtubule is a 1.5 start pseudo-helix with a seam (or a 3 start helix if α - and β -subunits are not distinguished). The protofilaments of microtubules with 10 and 16 protofilaments have a slight superhelical twist that is not indicated, and not all protofilaments at the sides are shown.

suggests possible mechanisms for how such closely related motors can move in different directions. For example, a conserved clockwise rotation of the head or an external subdomain relative to the microtubule would shift an attachment point on the left towards the plus end, while shifting an attachment point on the right towards the minus end. Alternatively, the direction of the secondary binding site could be important. In this regard it will be crucial to extend the resolution of the decorated structures so that inter-tubulin dimer contacts can be distinguished from intra-tubulin contacts along the protofilament.

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The polarity assignment should be more consistent within one laboratory, and the results of Hirose *et al.*⁵ indicate that the orientation is similar for different nucleotide states of kinesin and for *ncd*. This implies that the tilt differences reported by the three laboratories result from differences in the assignment of the polarity of the microtubule, and that both motors have similar orientations that do not change drastically with changes in bound

nucleotide. Hirose *et al.*⁵ do observe nucleotide-dependent changes in the angle of a possible attachment region that could represent the power stroke. These changes, however, are probably too small to produce 8-nm steps¹⁵ without amplification in a dimer of heads.

Further work with dimeric head constructs is needed to determine if the tethered head⁸ is preferentially directed to its next binding site and how the orien-

tation changes during the ATPase cycle. In this regard, rapid freezing and analysis of unstained samples as employed by Kikkawa and colleagues⁴ should be particularly useful for trapping of transient intermediates. □

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OBITUARY

Jonas Salk (1914–95)

JONAS Salk, who died on 23 June, was a medical hero throughout the world, and especially in the United States. He achieved that status on 12 April 1955, when it was announced that his vaccine was safe and effective in preventing poliomyelitis. In a country that had for many years been battered by polio epidemics almost every summer, affecting mainly children, the announcement meant that a great battle had been won. Salk became a kind of god.

The path that led to this achievement began in 1942, when Salk joined the department of epidemiology at the University of Michigan, where a vaccine against influenza, made from virus inactivated with formaldehyde, was being developed. At the end of the war Salk moved to Pittsburgh, and continued to work on the influenza vaccine. In 1949 he was approached by the head of the March of Dimes Foundation, which ran a highly successful fund-raising campaign to fight polio. Salk received a contract for identifying the various types of poliovirus, a necessary step for producing a vaccine. There were widely different opinions about how a vaccine should be made; the majority of scientists opted for one based on attenuated virus, believing that only persistent infection can lead to long immunity.

After Enders and colleagues showed that poliovirus can be successfully grown in cultures of monkey cells, a means became available for obtaining virus much more easily. Salk then took advantage of this technology, combining it with the knowledge he had gained about poliovirus and his experience with the influenza vaccine. He disregarded current scientific opinion, and prepared a formalin-inactivated polio vaccine.

The new stature gained by Salk in 1955 gave him the opportunity to realize a dream — to build an institute for basic biological research, separate from universities, and intended for the study of human nature in all its complexity. With the help of the March of Dimes (now aimed at research into birth defects), he built the new institute at La Jolla, California, near the developing campus of the

University of California. He was preoccupied as much with the external appearance of the institute as with its organization, and convinced Louis Kahn to design it; Salk contributed greatly.

In this intellectual venture, Salk found approval and support from outstanding figures of the time, such as Jacques Monod, Leo Szilard and Warren Weaver. They attracted several promising young people in various fields, and together formulated a plan for the institute that



included biologists, artists and philosophers in the first group of 'fellows'. The dream of the interaction between science and the humanities, however, did not quite work out in practice, and the institute rapidly became focused on biological research — becoming remarkably successful, especially in the fields of molecular biology and neuroscience.

The arrival of the AIDS epidemic gave Salk a new reason to pursue his vaccine work, again going against prevailing scientific wisdom. His fundamental and original idea was to use the vaccine to improve the conditions of people already infected, what he called a 'therapeutic vaccine', a concept that most specialists did not accept. He conducted this research until the last days of his life. Before he died he could see a slight

change in the scientific thinking and some acceptance of the vaccine he was proposing, and the initiation of limited clinical trials. But whether this line of approach will prove fruitful remains to be seen.

Salk was a very kind person of profound convictions, who would not be easily led by other opinions, although he listened to them. He persisted in doing what he thought was right, even in the face of strong opposition. He worked hard in pursuing both his research and his dreams. He had an optimistic, philosophical mind, which he revealed especially in books dedicated to the fate of mankind in which he indicated ways for a continuing evolution. He was a physician concerned about the health of the people of the world, especially in Third World countries; for instance, he convinced the Indian government to ensure that the 22 million children born in India every year receive the polio vaccine, possibly as a fourth vaccine added to the usual DTP (diphtheria–tetanus–pertussis). He provided help to get this accomplished.

For his work on polio vaccine, Salk received every major recognition available in the world from the public and governments. But he received no recognition from the scientific world — he was not awarded the Nobel prize, nor did he become a member of the US National Academy of Sciences. The reason is that he did not make any innovative scientific discovery. The fact that a fundamental advance in human health could not be recognized as a scientific contribution raises the question of the role of science in our society. Salk's research met many of the characteristics of good science, such as independence, originality in the pursuit of an unpopular goal, and ability to conclude successfully a project in which no one else believed. His dedication was complete and totally unselfish. It is true that he did not contribute any technological advance; but is science only technology? Renato Dulbecco

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Postmodern complexes

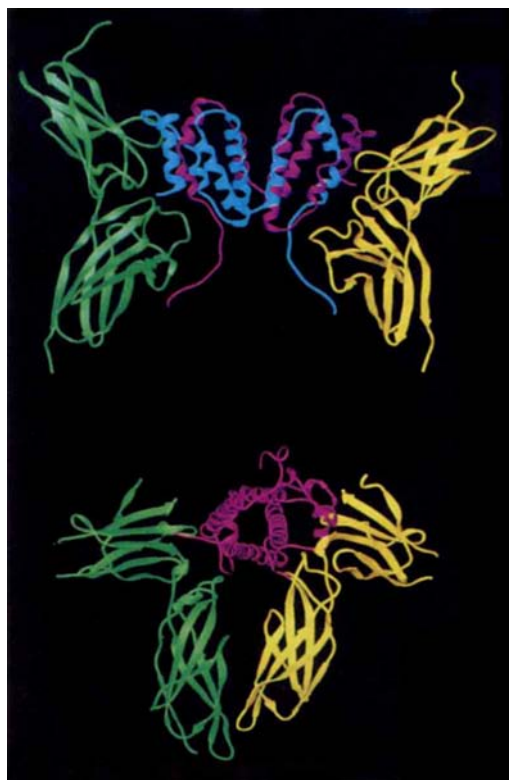
J. Fernando Bazan

It has become maddeningly routine to open a journal and view, crammed into a few pages of dense script and colourfully ribboned images, yet another molecular structure more magnificent and full of promise than the last. This exhilarating pace of structural discovery in biology continues with the unveiling of a provocative new receptor-ligand complex fold by Walter *et al.* on page 230 of this issue¹. Just over three years ago, the crystal structure of growth hormone (GH) bound to its dimeric receptor complex provided the first detailed glimpse of a cell-surface cytokine signalling complex². The classical lines of this prototype fold are still discernible in the architecture of interferon- γ (IFN- γ) bound to its primary receptor (IFN- γ R α), but striking new designs in the present complex¹ compel a revamping of ideas about how cytokines interact with haematopoietic receptors.

The X-ray study of the GH-receptor (GHR) fold² cemented the notion that the binding domains of haematopoietic receptors had a common tertiary structure consisting of tandem immunoglobulin (Ig)-like modules³. Likewise, a rapidly growing number of cytokine structures determined by X-ray and NMR display the four-helical bundle topology of GH, with particularly close agreement in two core helices (labelled A and D) that form the principal binding epitopes in the receptor complex^{2,4,5}. With their respective amino-terminal Ig modules splayed outwards at 85°, the GHR dimer forms a loop-rich trough where the helical GH ligand is docked in a prone position, one receptor being involved in a high-affinity interaction with (mostly) GH helix D and a contiguous AB loop, the other in a less extensive contact with the packed surfaces of GH helices A and C; the receptor subunits also touch in a glancing sheet-to-sheet interaction close to the membrane (see bottom structure in the figure)². The cardinal features of this picture were expected to recur in homo- or heterodimeric receptor binding complexes involving helical cytokines^{2,4,5}.

The oligomerization of IFN- γ and soluble R α molecules leads to a somewhat different situation (see top structure in the figure)¹. IFN- γ exists in solution as an intertwined dimer with each helix-bundle module composed of chains from both subunits; the crossover links create an elbow angle between bundles of about 72° (ref. 4). As a result, the V-shaped IFN- γ dimer displays two symmetrically opposed pairs of helices (A and F in ref. 1) equivalent to GH helices A and D (ref. 4). Each molecular dimer then binds two high-affinity R α chains mainly using the F

helix and nearby AB loop portions of the respective bundle faces¹ — the bound receptors are separated from each other by a remarkable 27 Å. When displayed so that the two-fold axis of the IFN- γ dimer is perpendicular to the putative cell membrane (top in the figure), the individual helix bundles appear to be almost upright when docked to their respective receptors; this is partly an illusion, as the R α molecules are tilted at 60° relative to the vertical, but the IFN- γ subunits remain at



Comparison of the crystal structures of (top) the IFN- γ dimer bridging two IFN- γ R α chains¹, and (bottom) growth hormone bound between identical GHR subunits². Graphics kindly provided by M. R. Walter using the program RIBBONS (M. Carson).

about a 60° angle away from a GH-like prone position¹.

A reason for this drastic redirection of the cytokine from the R α binding cleft is the 40° change in relative domain orientation between component Ig modules from the prototype GHR fold¹. The 125° angle between Ig structures is instead closest to the conformation of the tissue factor (TF) monomer^{6,7} and is possibly a constant feature of the structures of all IFN-like (or class 2) receptors of the haematopoietic superfamily (as opposed to distantly related class 1 or GHR-like molecules)³. Tissue factor is closely related in sequence to IFN receptors but instead serves as a membrane-bound cofactor for the pro-

tease factor VII in the coagulation cascade³; fortuitously, we can consider the TF structure^{6,7} as an unliganded IFN receptor fold — superposition of IFN- γ R α and TF shows surprisingly close global accord, probably indicating a ligand-insensitive quality of such receptors. The more relaxed attitude of Ig domains in the IFN- γ R α and TF structures then decreases the number of exposed, potentially ligand-interacting loops at the domain interface relative to the GHR¹. In addition, class 2 Ras (and TF) exhibit a large chain insertion between the second and third β -strands of the receptor carboxy-terminal Ig module³ that forms an extravagant loop structure protruding into a prospective GHR-like trough¹, and instead buttresses one face of the more upright, docked cytokine (top in the figure). The factor VII-binding site of TF, however, has migrated sideways to a β -sheet surface^{6,7}.

The topologically equivalent binding epitopes on GH and IFN- γ encounter residue constellations from distinct receptor loops that nonetheless remain remarkably similar in composition and function¹. A prevalence of Trp and Tyr aromatics ringed by charged Arg, Lys, Glu or Asp amino acids is also seen at the heart of the low-affinity contact site between GHR and GH², and in an alternative pairing of the prolactin receptor and GH⁸. Clackson and Wells⁹ have shown that contact surfaces on both ligands and receptors have two distinct, complementary zones: a small functional patch embedded in a larger structural epitope. Amphipathic residues such as Trp are favoured in the functional core for they provide conformational plasticity with their aromatic rings that mould themselves to the interacting surface and achieve a flush hydrophobic fit^{9,10}. Charged side chains at the periphery of functional cores are often found

stacked in parallel against these and other aromatic rings^{1,2,8} which still permit the terminal polar groups to influence the specificity of interaction through electrostatic effects or intermolecular hydrogen bonds^{9,11}. Therefore, within the same structural epitope, alternative, perhaps even overlapping, functional sites can guide binding to different targets^{2,8,9} — a commonplace occurrence with promiscuous cytokines and receptors in the haematopoietic system^{2,4,5,8} or antigen-combining sites in antibodies^{10,12}.

Comparison of receptor-bound and free IFN- γ reveals that the helical scaffold is fairly rigid save for a flexible AB loop segment that forms a 3₁₀ minihelix in the

bound form¹. This loop adaptation mirrors the AB loop changes seen in GH between the free (affinity matured) structure, or two distinct bound states^{2,8}. Another notable eccentricity of IFN- γ binding concerns the role of a crucial basic cluster at the carboxy terminus of a long, curving tail; this structure can be modelled to nestle against a conserved acidic patch on the R α carboxy-terminal Ig domain¹, which curiously exists mostly because of an IFN- γ R α -unique³, disulphide-looped chain extrusion¹. The 2:2 IFN- γ :R α structure remains an intermediate, inactive complex in wait of one or two accessory R β molecules¹³ that speculatively dock against respective R α s in the vicinity of the same acidic patch, perhaps engulfing the basic IFN- γ tail¹. This potential R β binding model would also engage the A-C helix faces of each IFN- γ helix bundle and hence locally reconstitute a bioactive, postmodern version of the classical GHR binding trough¹.

The crystal structure of dimeric interleukin (IL)-10 bears an extraordinary resemblance to the IFN- γ fold, with just a slightly greater elbow angle of 97° between intercalated helix bundles (ref. 14 and M. R. Walter, personal communication). IL-10R α is squarely in the class 2 cytokine-receptor camp^{14,14}, so the structural design of intermediate and signalling IL-10 complexes should closely mirror the IFN- γ case. In contrast, IFN- α/β types are not apparently dimeric in solution, but do associate with the requisite R α and R β subunits to form 1:1:1 heterotrimeric signalling complexes¹⁵ which could best resemble a single IFN- γ or IL-10 helix bundle:R α :R β module and not the prototype 1:2 GH:GHR ensemble¹⁶.

The nucleation of hexameric receptor-cytokine signalling complexes, as is confidently expected for IFN- γ and IL-10 (with concomitant implications for intracellular signalling^{13,15}), has given physical form to an emerging trend in the haematopoietic system. The crystal structures of leukaemia inhibitory factor (LIF)⁵, ciliary neurotrophic factor (CNTF)¹⁷ and a robust model of IL-6 (ref. 18) fold very similarly to GH but display a puzzling three-dimensional patchwork of receptor epitopes that overwhelm the simple, two-site binding model of a stand-alone GHR-like dimer complex. A higher order 'complex of complexes' parsimoniously solves this quandary for IL-6 in a 2:2:2 association with IL-6R α and gp130 (ref. 18) and provides a pleasing model for LIF, CNTF, oncostatin-M, cardiotrophin, IL-11 and IL-12 multimeric signalling complexes^{5,18}.

However, for other helical cytokines that are tied together as solution dimers⁴ and might be expected to act as IFN- γ -like aggregators, the evidence is to the contrary. The primary receptor epitope on

IL-5 straddles the interlaced dimer⁴ interface so that the D-helix binding of a first R α subunit is a symmetry-breaking event that sterically occludes the other R α docking site, and forces the choice of a contrary, single R β A-helix site¹⁹. On the other hand, the disulphide-bridged macrophage colony-stimulating factor (MCSF) dimer⁴ does act as a bridge, but only between a pair of identical fms/CSF-1 tyrosine kinase receptors (not superfamily molecules) which each bind a symmetry-related A-helix site per monomer using Ig domains²⁰.

Walter and colleagues' structure¹ of the IFN- γ R α complex* offers a new organizing principle for class 2 cytokine receptors, and a welcome lead to understanding the greater associations of a large segment of the class 1 receptor family. The two cytokine-binding geometries exhibited by the GHR² and the IFN- γ R α ¹ hopefully signal two extremes, but the latitude between them is great — modellers should beware of blindly using one or the other as a template¹⁶. Nonetheless, the basic rules underlying the molecular recognition of IFN- γ or GH by disparate receptors seem to be reassuringly similar. Allusions and cautions aside, it remains gratifying to see how rapidly this field is being propelled by force of its protein structures. On to the next! □

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*I have learned that a similar X-ray structure has just been solved using MAD phasing by S. Ealick, F. Winkler and co-workers at Cornell University and Hoffman-La Roche (ACA Abstr. W130, Montreal, July 1995).

Silent intervals

We all hate unexpected loud noises. Sudden crashes and explosions can startle us badly, and even damage our ears. Daedalus now has an answer.

His first idea was a sort of powered flap which snapped over the ear canal on command. Being so close to the ear, it made more noise than the sound it was keeping out. He then recalled those electro-rheological fluids, typically suspensions of fine particles in an insulating oil, whose viscosity rises in an electric field. The particles each acquire a dipole, and clump together into a tenacious structure which disperses when the field is turned off. It all happens in milliseconds, and in total silence. Daedalus is now using such fluids in a silent, fast-acting, electrically controlled earplug.

The prototype is a little hollow tube blocked by a succession of diaphragms soaked in a suitable electro-rheological fluid. With the field off, the diaphragms are perfectly flexible and sound travels down the tube as if they weren't there. But when the field is on, they lock up into stiff, highly damped barriers which reflect most of the sound and absorb the rest. The earplug will be wired to a small control box that nestles behind the ear like a miniature hearing aid. This in turn will get its orders by short-range radio.

The primary market will be military. To 'silence' a gun or grenade, a little transmitter will be attached to it, so that it sends out a radio pulse just before it goes off. Next time the special forces storm some luckless gang of terrorists, firing guns and throwing stun grenades, they will do so in blissful, contemplative quiet. Their radio earplugs will neatly clip out the noise of their own explosions, while leaving their ears open for the quiet exchange of orders and the panicky cries of their victims. Meanwhile, the victims will be deafened and disoriented by the stunning racket of the assault.

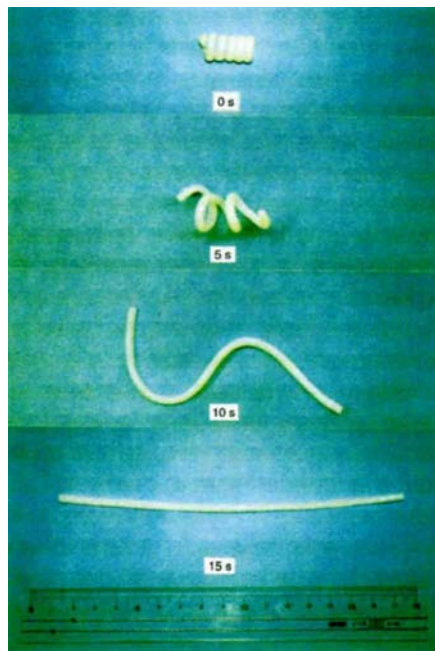
A simpler, less selective arrangement could exploit the slow speed of sound. A tiny microphone on a stalk a few centimetres away from the ear could detect an approaching bang, and switch on the earplug before the bang reached it. Such pre-emptive earplugs would be widely welcomed by boiler-makers, engineers and the parents of clumsy or hyperactive children. They might best be tuned not to exclude loud pulses, but merely to reduce them to a steady level. Pop music, that universal annoyance, could be tamed in this way. Normalized to one intensity, deprived of all amplitude modulation, it will lose its insistent beat. It will be reduced to a rhythmless drizzle of relatively unobtrusive frequency-modulated noise.

David Jones

Shape memory in hydrogels

SIR — The ability of many polymer gels to modify their shape in response to external factors^{1,2} (such as temperature, pH, electric fields and light) may find biomedical application. Of particular interest are polymer hydrogels, in which water molecules fill the interstitial sites of a crosslinked polymer network. Unlike metals, these materials are 'soft' and 'wet', which might improve their compatibility with biological tissue³. We have discovered a new phenomenon in polymer hydrogels — a 'shape memory' effect — which could form the basis of a thermally responsive diaphragm, a temperature-activated surgical clamp or a thermal actuator with gentle action.

A material is said to show shape memory if it can be deformed to a new shape (which it retains), only to revert to its original shape when heated above a certain critical temperature. Perhaps the most famous example of such a material is titanium–nickel alloy, produced by a reversible martensitic structural transformation⁴. The mechanism in our polymer gels is very different.



An example of shape memory. The gel was prepared by radical copolymerization at 50°C for 24 h. The total monomer concentration in ethanol was kept at 3.0 mol dm^{-3} in the presence of $3.0 \times 10^{-2} \text{ mol dm}^{-3}$ *N,N'*-methylenebis(acrylamide) and $3.0 \times 10^{-2} \text{ mol dm}^{-3}$ α,α' -azobis(isobutyronitrile) used as an initiator. The copolymer gel was formed in a straight glass tube of diameter 5 mm and then swollen with water (dry samples do not show the shape memory effect). The gel was then heated to 50°C, coiled and then cooled to room temperature. The gel is rigid and retains its coiled shape (top panel). On heating again to 50°C, the gel becomes soft and recovers its original, straight shape.

We have shown previously⁵ that the mechanical properties of water-swollen gels prepared by copolymerizing acrylic acid and *n*-stearyl acrylate are strongly temperature-dependent. Below 25°C, the gel behaves as a hard plastic. But if the temperature is raised above 50°C, the gel becomes very soft and can be stretched to at least 1.5 times its original length. When the swollen gel is heated, its Young's modulus decreases by about three orders of magnitude, from 10^7 dyn cm^{-2} at 25°C to 10^4 dyn cm^{-2} at 50°C. These drastic changes in the mechanical properties of the gel were ascribed (on the basis of small-angle X-ray diffraction analyses)⁴ to a reversible order–disorder transition associated with the interactions between the alkyl side chains of the stearyl acrylate units. Below 50°C, the long alkyl chains adopt a crystalline aggregate structure, which renders the material mechanically robust. Above this temperature, the packing of the side chains is amorphous and the material becomes soft and flexible. We suggest that the same mechanism causes the shape memory effect in these gels.

Although we call this the shape memory

effect, we emphasize that the shape that the gel 'remembers' is the one in which it was originally formed. If the gel is heated above the transition temperature (50°C) into its 'soft' state, it can be readily deformed and stretched to a new shape, but if the gel is then cooled while holding it in its deformed state, it becomes rigid and retains its new shape even after the deforming loads have been removed. We suggest that it is the formation of crystalline aggregates among the side chains that locks in the new shape. If the unloaded, deformed gel is then once again heated above the transition, it recovers its original size and shape after a few seconds. This behaviour is illustrated in the figure.

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Costs of conception in baboons

SIR — Packer *et al.*¹ reported that miscarriage occurred more frequently in high- versus low-ranking female baboons at Gombe National Park. We obtained a comparable result at Mikumi; confirmed abortions among females of high- to low-dominance rank quartiles comprised 15, 13, 8 and 3 per cent of all pregnancies, respectively (number of pregnancies = 47, 48, 37, 38; $P < 0.05$, logistic regression). Here I provide data to suggest that the higher miscarriage rate results from a less socially and physiologically constrained conception rate among high-ranking females. Their conception appears to be less socially constrained because survival of offspring born to dominant females is much less affected by their time of birth in relation to others^{2,3}. Dominant females accordingly conceive more readily^{1,3} at lower progesterone concentrations than do low-ranking females. These hormone con-

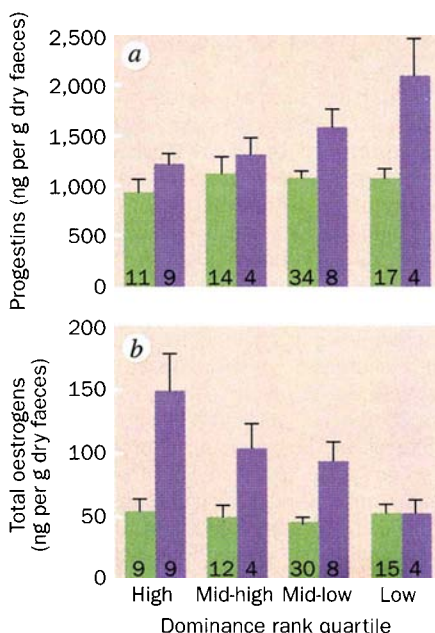
centrations are sufficient for high-ranking females to establish implantation, but may not always be sufficient to maintain it.

I collected fresh faecal samples between 1990 and 1992 from 30 individually recognizable adult female baboons of proven fertility in two separate troops at Mikumi National Park, Tanzania. I analysed samples for progestins and oestrogens (progesterone and oestradiol metabolites, respectively)^{4–7}, because early luteal-phase progesterin and oestrogen concentrations are positively correlated with successful implantation in baboons^{8,9} and humans^{10,11}.

Mean luteal-phase concentrations of progestins and oestrogens were significantly higher in conceptive versus non-conceptive cycles (see figure). Mean luteal-phase progesterin concentrations during conceptive cycles were significantly lower in females of high- versus low-dominance rank, but did not differ by rank

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Mean luteal-phase (post-ovulation days 4–11) progesterin (a) and total oestrogen (b) concentrations in faeces of conceptive (purple) and nonconceptive (green) cycles as a function of female dominance rank. Post-ovulation day 4 corresponds to the day in which progesterin and oestrogen concentrations in conceptive cycles became significantly different from those in nonconceptive cycles. Post-ovulation day 11 corresponds to the time of implantation¹⁵, given the 36-h time lag between steroid secretion in blood and excretion in faeces⁵. Mean luteal-phase progesterin and oestrogen concentrations were significantly higher in conceptive than nonconceptive cycles ($P < 0.0001$ in both cases), as well as significantly associated with dominance rank ($P < 0.04$ and < 0.02 , respectively). The interaction between pregnancy and dominance rank was also significant for oestrogens ($P < 0.03$; ANOVA). Rank was quantified based on the outcome of bouts of aggression systematically recorded¹⁶. Sample sizes are shown in bars. Subjects were fully habituated to human observers, having been studied almost daily since 1974. Faecal samples were obtained from each female approximately 1–3 times per week, depending on reproductive condition. Samples were stored, extracted and assayed for progesterins and oestradiol using the methods in refs 4–7. Total oestrogens were measured using the RSL ¹²⁵I total oestrogen kit (ICN Biomedicals, Costa Mesa, CA). Every attempt was made to include only ovulatory cycles in this study, based on a transient rise in luteal-phase progesterin concentrations. Ovulation was presumed to have occurred 2 days before onset of sex-skin detumescence¹⁵. All conceptive cycles in this study ended with a birth. Some cycles classified as nonconceptive undoubtedly represented early abortions. The 17 miscarriages described in the text occurred before the hormone study.



during nonconceptive cycles (a in the figure). The dominance-related faecal oestrogen pattern was opposite to that of faecal progesterins (b in the figure). Oestrogen concentrations during conceptive cycles were greatest in females of high- versus low-dominance rank, while showing no rank effect during nonconceptive cycles.

The rank-related progesterin patterns in conceptive cycles appear to be tied to oestrogen-mediated changes in progesterone receptor densities. Oestrogen increases progesterone receptor densities in the epithelial layer of the endometrium (where implantation occurs), but has little effect in the deeper layers (stroma and myometrium) that maintain pregnancy in Old World primates^{12–15}. Progesterone has the opposite effect, decreasing progesterone receptor densities in the epithelial layer.

These findings suggest that the higher oestrogen concentrations observed in the early luteal phase of dominant females (b in the figure) may be dampening the progesterone-induced decline in progesterone-receptor densities over the early luteal phase. The resultant higher receptor density should make implantation easier to achieve in dominant females with less progesterone present. However, the lack of similar receptor-density changes in the stroma and myometrium seems to compromise the ability of this lower progesterone in dominant females to sustain the pregnancy. Subordinate females would experience the opposite: based on

oestrogen concentrations (b in the figure), their progesterone receptor densities would be lower, requiring more progesterone for implantation, which also should improve their ability to sustain pregnancy (a in the figure).

In conclusion, high-ranking females can afford to conceive more readily because their offspring are more buffered from the social conditions in which they are born. However, the physiological mechanisms that facilitate these conceptions seem to bear a cost of increased susceptibility to miscarriage.

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Hot PUMPing plants ?

SIR — In their Scientific Correspondence, Vercesi and colleagues¹ suggest that the presence of PUMP, an uncoupler-like protein in potato tuber mitochondria, may correspond with the absence of the alternative oxidase in this tissue. But is the alternative oxidase thermogenic; does the location of PUMP correspond with the absence of the alternative oxidase; and

does PUMP fulfil an analogous role?

The alternative oxidase is responsible for heat production in thermogenic lilies², but in this system heat production is linked to a massive increase in enzyme activity concurrent with a complete decrease in cytochrome oxidase activity^{2,3}. There are no reports linking alternative oxidase activity to thermogenesis in other plants. Why should a plant need this heat production mechanism? At the levels of activity reported for the alternative oxidase (about 100 nmol O₂ per mg mitochondrial protein per min) in plant tissues, the heat change would be very small compared with the rate that can be achieved in thermogenic lilies (2 μmol O₂ per mg mitochondrial protein per min)⁴. The alternative oxidase is implicated in various processes, such as wounding, pathogen attack, elevated carbohydrate status and addition of salicylic acid⁵, but none of these requires thermogenesis.

Is the presence of PUMP correlated with the absence of the alternative oxidase? Although potato tuber mitochondria do not naturally exhibit alternative oxidase activity, it can be easily induced in these tissues⁶, showing that they can express this enzyme.

Do PUMP and the alternative oxidase function in an analogous role? The alternative oxidase has been implicated in various processes. Although its exact role is unclear, it does function to oxidize reduced compounds without the production of ATP. Rather than this function being related to the generation of heat, it is more easily envisaged as being related to the diversion of metabolites in the Krebs cycle to other biosynthetic functions⁵. The uncoupled oxidation of reduced compounds allows the cycling of cofactors necessary for glycolysis and the Krebs cycle and so keeps catabolism at a high rate⁷. Under such conditions plants will be synthesizing a number of compounds for growth and development. The control of alternative oxidase activity by two separate biochemical parameters⁸ ensures that this activity is highly regulated in reference to the metabolism of the cell/plant. In my view, there is no evidence to suggest that the PUMP protein has this role.

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Deep sea thrills

Charles H. Langmuir

Explorations: My Quest for Adventure and Discovery Under the Sea. By Robert D. Ballard with Malcolm McConnell. *Hyperion*: 1995. Pp. 407. \$24.95.

UNDERSEA exploration by submersible is one of the most captivating aspects of oceanography. Working in an environment more hostile than outer space, penetrating depths far greater than those accessible to military submarines, seeing parts of the Earth previously unseen by human eyes, discovering new life forms or ancient wreckages — how could anyone fail to be fascinated? Nonetheless, a particular talent is needed to tell the tales of deep-sea exploration in a way that keeps the interest of a lay audience. Who better to undertake this task than Robert Ballard, the man who led the expedition that discovered the wreck of the *Titanic* in 1985?

Ballard began his career as a naval officer in 1967, serving as the funding conduit between the US Office of Naval Research and Woods Hole Oceanographic Institution on Cape Cod, Massachusetts. *Alvin*, the Navy's three-man research submarine, was then already Woods Hole's pride and joy, and Ballard took a particular interest in its operations. While preserving his naval connections as a reserve officer, Ballard became a graduate student at Woods Hole. Exploiting his naval connections to the full, and with abundant energy, Ballard had the knack and good fortune to be in the right place at the right time, and has participated in many of the most important investigations of the deep sea over the past 20 years. Here, aided by a professional writer, he relates his experiences with a sense of salesmanship that can make even the mundane seem exciting. The result is a book that is difficult to put down, for it possesses many characteristics of a modern thriller.

As a thriller it is successful. The story begins in 1963 with a vivid account of the loss of the US nuclear submarine *Thresher* and the death of its entire crew as it sinks in 8,500 feet of water. It then jumps forward 20 years, with Ballard and his team mapping the disaster site using an undersea camera system. During this expedition, Ballard professes to have "found the Rosetta Stone that would decipher the mystery of deep ocean wrecks". Next comes the successful conclusion of the search for the *Titanic*, "the Golden Fleece of undersea exploration". In the first 20 pages we have been carried skilfully from disaster to triumph, and it is clear we are reading more of an adventure story than a routine scientific autobiography.

The professional scientist may take a sceptical view of Ballard's literary liberties. Ballard often takes routine events in

the life of a sea-going scientist and spins a yarn around them to create high drama. In recounting his PhD thesis defence, he has the reader believing that a single question from the audience could have put his career in jeopardy. He describes the recovery of a camera sledge onto a ship as if the weather were exceptionally dangerous, whereas a photograph of the same episode shows normal seas. Hyperbole



LIVING oasis — red-blooded worms emerge from their tubes near the chimney of a 'black smoker', as seen by *Alvin* in 1979 on the East Pacific Rise. The picture is taken from *The Mid-Ocean Ridges: Mountains Below Sea Level* by Adolphe Nicolas, a clear and well illustrated introductory text expressing the author's personal viewpoint. Springer, DM58, £25 (pbk).

makes the story and man larger than life: the deep sea poses a "relentless, unforgiving menace"; a ship's engines are "powerful thrusters"; the van with routine technical equipment is a "a nerve center crammed with electronics"; and the camera sledge (called "Argo") is "a vehicle of exploration as audacious as its namesake in classical Greek mythology". The narrative is often carried forward by dialogue, as if the events were unfolding on the printed page. Despite sometimes being contrived and over the top, the literary mechanisms work: they bring the discoveries to life and captivate the reader's interest.

Although it is certainly a rousing good adventure story, the book must also be considered as a scientific autobiography, because it refers to the historical facts of discovery and presents the evolution of a man and his scientific career. Ballard compares his life to the epic voyages of a mythological hero, as enunciated so eloquently by Joseph Campbell. The outline and chapter headings of the book

follow Campbell's heroic paradigm, from early questioning, through great trials, to ultimate truth shared with the world. Ballard names his equipment "Argo", "Jason" and "Medea". In recounting how he was asked to present the case for submersibles to an early planning meeting for the FAMOUS project (a French-US venture that provided the first detailed examination of a mid-ocean ridge), Ballard portrays himself as a young hero, not yet a PhD, under the stern glare of the "Gods of Oceanography" set in opposition against him. After much discussion at the meeting, the submersible idea prevailed. Ballard concludes that "*Alvin* and I were given the chance to participate in one of the most important scientific expeditions of the century". This tendency towards

self-glorification, apparent in the book's subtitle, and the deprecatory attitude towards many scientific collaborators, many of whom were the principal authors on subsequent scientific publications, detract from the historical value of the book.

The epic paradigm may work well for readers willing to accept Ballard as the conquering hero of the deep. Others less smitten may wonder about true heroism, particularly when Ballard describes how he handled the difficult interpersonal problems that are a part of any life. As Campbell emphasizes, the classic voyage of the hero is ultimately an inner journey, where wisdom is gained through suffering. Gilgamesh, through a moment of inopportune sleep, loses the elixir of eternal life before he returns to his kingdom, externally empty-handed. Humility before life's power, recognition of good fortune as a gift rather than an egotistical accomplishment, a sense of being part of rather than above humanity — are not these essential

elements of the hero's journey? They are missing from Ballard's book.

Ballard reveals himself as a superb salesman, capable of delivering a glamorous view of sea-going exploration as mythic accomplishment. In this sense, he provides a useful public service: few scientists can convey their subject or the events in their life in such a gripping way. Those interested in deeper questions of discovery and the nature of man and science will need to look elsewhere. □

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Optimistic mumblings

Stuart Sutherland

Speaking Minds. Edited by Peter Baumgartner and Sabine Payr. Princeton University Press: Pp. 342. \$29.95, £24.95.

SPEAKING MINDS records 20 interviews with workers in cognitive science: it was not of course possible to include all the most eminent ones — some had to be omitted in order to write blurbs for the cover. The representation of the disciplines that make up the subject is odd: while there are eight philosophers and nine workers in artificial intelligence (AI), there is only one lonely experimental psychologist. The preponderance of the former disciplines in the book has determined the issues discussed. It is evident

that the problems posed by philosophers are insoluble, whereas many of those speaking for AI are falsely convinced that they already hold the solution to understanding the mind.

On one thing all are agreed: cognitive science is about understanding human intelligence. The contributors are, however, cautious about strong AI — the idea that a sufficiently intelligent computer program would be a mind or would even be conscious. The emphasis on intelligence may arise because the contributors are themselves intellectuals. No computer program could remotely resemble a person unless it had drives and emotions and, as Joseph Weizenbaum pointed out many years ago, such emotions could not be real — no computer could genuinely feel lust, anger or hunger. Moreover, the AI fraternity forgets that most people most of the time think and act in highly irrational ways. Nobody has attempted to incorporate these human failings into a computer program.

Some contributors such as Herbert Simon proceed in a top-down manner to infer from behaviour the mechanisms subserving it; others such as Patricia Churchland favour a bottom-up approach, starting with the functioning and organization of nerve cells. But so far neuroscience has thrown singularly little light on cognition, although it has sometimes confirmed theories, put forward by psychologists, as in the case of colour vision, and it has occasionally given us clues about how certain perceptual tasks such as object recognition are performed. Moreover, however much progress neuroscience makes, there will always be a need for higher-level theories, if only because the human mind can

process only about seven ideas at any one time. A diagram of the connections made by all the nerve cells in the brain would be uninterpretable.

The book's main theme is the debate between proponents of classical AI (based on manipulating symbols) and those of connectionism — the construction of networks in which layers of units are randomly connected to those of the next higher level with the strengths of the connections changing as the input units are triggered. Changes can also be determined by making the output for a given input correspond more and more closely to the one desired ('back propagation'). Learning is represented by changes in connectivity spread throughout the network: there is no clear-cut symbolization. Connectionists argue that their system is plausible because, like the brain, it works in parallel; it generalizes and discriminates as people do without specific feedback, and failure of several units does not totally impair previous learning; they also argue correctly that the brain cannot for the most part use serial processing. The advocates of classical AI retort that thinking is a serial process; that connectionism cannot solve problems (such as parsing) based on processing symbols according to rules; that a classical architecture can be operated in parallel; and that back propagation is an implausible method, given what we know about the nervous system. The latter point is doubtful because learning many tasks, such as how to ride a bicycle, needs knowledge of results. It is even possible that those mysterious recurrent pathways are there to prolong the existing excitation so that the connections that carry it can be strengthened if the right result occurs. Neither approach can begin to explain why the organization of neurons should be so remarkably similar in all neocortical areas, despite the fact that they serve completely different functions.

There is a good deal of dogmatism in the book — few contributors attempt to support their views with concrete examples. Paul Churchland announces bluntly that "Our current neural theory explains qualia surprisingly well". Again John Searle claims that computer programs only have syntax and therefore cannot have intentionality, but he does not meet the standard objection, cogently expressed here by Simon, that they could be said to have semantics if they interacted with the world.

One has the feeling that the interviewer asked the wrong questions. It is surely time that the Turing test and Searle's Chinese room were put to rest — neither helps us to understand the mind. Moreover, the contributors were not asked what they regard as the important achievements of cognitive science. Instead they were asked about its future, a ques-



COLLISION of comet Shoemaker-Levy 9 with Jupiter in July last year. From the cover of *The Quest for Comets* by David H. Levy, now out in paperback. For a review see *Nature* 369, 716 (1994). Oxford University Press, £7.99.

tion that produced, as one might expect, optimistic mumbling. Apart from Daniel Dennett's slovenly chapter (he begins: "Before there was a field called Cognitive Science, I was working in it"), Peter Baumgartner and Sabine Payr have produced a well written book, although it is more amusing than informative. It is interesting to observe the reluctance of the great to change their minds and to note the ways in which their arguments pass one another by. □

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Thought transference

Ray Monk

Wittgenstein Reads Freud: The Myth of the Unconscious. By Jacques Bouveresse. Princeton University Press: 1995. Pp. 143. £15.95, \$19.95.

PERHAPS the strangest remark Wittgenstein ever made (among, it must be said, some stiff competition) was his comment to his friend Rush Rhees that he considered himself to be a "disciple of Freud". Given that Wittgenstein seems to have read only two or three of Freud's books, that he showed little interest in — and even a certain hostility to — psychoanalysis and that almost all his few recorded comments on Freud's work are sharply and often devastatingly critical, one wonders what he meant. With disciples like this, one feels, who needs detractors?

One searches in vain in Wittgenstein's writings for an extended discussion of Freud's work and influence. His remarks about Freud are, rather, scattered throughout his rather messy corpus: a few paragraphs in *Culture and Value* (the collection of his "non-philosophical" writings

selected and edited by his executors); some allusive comments in the notes of his lectures made by his students; a few remarks in his recorded conversations with his friends Maurice Drury and Oets Bouwsma; and the odd mention of Freud while discussing entirely different subjects in various of his philosophical writings, including *Philosophical Grammar* and *Remarks on the Philosophy of Psychology*. If there is a central text it is the "Conversations on Freud" recorded by Rhees and included in *Lectures and Conversations* published in 1966, but even this amounts only to a few pages of sketchy comments. If one were to gather together every mention of Freud by Wittgenstein and put them all in a book entitled, say, *Wittgenstein on Freud*, it would be a rather slim volume. (*Wittgenstein on Russell*, *Wittgenstein on Frege*, even *Wittgenstein on William James*, would, by contrast, be enormous.)

What, then, did Wittgenstein mean by describing himself as Freud's disciple? A common approach to this question is to see in Wittgenstein's 'therapeutic' method of philosophy important affinities with the techniques of Freudian psychoanalysis. When this line was taken by A. J. Ayer in an article in the *Listener* in the 1940s, however, Wittgenstein reacted angrily against it, insisting vehemently that the two were "different techniques". Another approach, and one that has gained increasing favour in recent times, is to emphasize the importance of finding the right metaphor that lies at the heart of both Wittgenstein's work and (on Wittgenstein's understanding of it) Freud's. "It's all excellent similes", Wittgenstein once told a class about Freud's work, echoing the remark he once made summarizing his own contribution to philosophy: "What I invent are new similes".

Jacques Bouveresse, in this meticulously researched and closely argued study, seems curiously unpuzzled by the enigma of Wittgenstein as a 'disciple' of Freud, and apparently satisfied to repeat the standard lines on the subject. His interest, rather, is in presenting Wittgenstein as a particularly trenchant and devastating critic of Freud. In this he follows other distinguished commentators, including Frank Cioffi and Thomas Szasz, and, despite the wealth of scholarship he brings to his task, he adds disappointingly little to what has already been said. His conclusion, for example, that "Freud defends a kind of classical scientific rationalism, while Wittgenstein clearly belongs to quite another order of thought" is by now little more than a commonplace, and his thesis that "the philosopher isn't really dealing with a science, while the mistake of psychoanalysis is essentially to believe that it is one" has been advanced so many times that it needs a more original defence to breathe new life into it.

One might have expected a French



Wittgenstein: "disciple of Freud".

philosopher to try to link Wittgenstein's criticisms of Freud to the use made of Freud's work by leading French thinkers, such as Lacan and Derrida. But Bouveresse self-consciously distances himself from the tradition of thought that prevails in his home country and instead allies himself unreservedly with the opposing Anglo-American analytical tradition, even to the extent of repeating its prejudices against the French ("we French are well known for our tendency to sometimes confuse the practice of philosophy with the practice of free association"). On Lacan, he has a few pages making short work of his notorious notion of the "language of the unconscious", whereas on Derrida he is altogether silent. (Vincent Descombes, on the other hand, devotes most of his foreword to a discussion of Lacan and Wittgenstein.) Most of Bouveresse's references are to leading Anglo-American philosophers-of-mind such as Donald Davidson and Daniel Dennett and to the huge body of Wittgensteinian secondary literature written in English.

To an English reader schooled in the literature, then, the book offers few surprises. Its discussion of the difference between reasons and causes, of "the problem of the reality of the unconscious" and of "the generalizing impulse" of philosophers seem, indeed, almost wearisomely familiar, and its criticisms of Freud look — especially in the wake of the recent wave of strident Freud-bashing — like attacks on an increasingly threadbare straw man. Nevertheless, in drawing together most of the remarks made by Wittgenstein on Freud, many of the relevant passages from Freud's work and a good deal of quotation from the secondary literature on the subject, Bouveresse has performed a valuable service for the Wittgenstein scholar. □

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Mary Evans

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REASONS

Freud: "threadbare straw man".

Expert advice

Giovanni F. Bignami

Idee per il governo: La ricerca scientifica. By C. Bernardini *et al.* Laterza, Italy: 1995. Pp. 117. L15,000 (pbk).

CAN advice be given graciously but explicitly to governments? Do politicians actually need ideas? Are they broad-minded enough to consider and even accept policy proposals, well reasoned and documented, from acknowledged experts?

One of the most prestigious Italian publishers, Laterza, is tackling these challenging questions in a series entitled *Idee per il governo*, the government in question being, of course, the current Italian one. This, the second volume in the series, is dedicated to scientific research (the first volume dealt with schools). Carlo Bernardini, professor of physics at the University of Rome, is one of the most charismatic figures in Italian science, as well as in the analysis of the role of science in modern society. In the opening 50 pages, he presents a typically concise yet complete view of the status of Italian research, setting it against that of other nations and in its immediate historical context. For the first time we see an explicit denunciation of the disastrous 'Berlusconian' attitude towards basic research. (Well versed in the use of such terms as 'Thatcherism' and 'Thatcherite', readers of *Nature* should have no difficulties with this rather ugly neologism.) Adopted in 1994 for only a mercifully short period, the attitude is likened here to "burning precious, antique furniture for heating the house": this might be a dramatic necessity, but should not hide a lack of ability to search for more appropriate firewood.

As is to be expected, data are plentiful, interesting and accurate. They are taken from the recent work of one of the experts called in for the wider discussion later in the book: Mario De Marchi's "Public Support of Basic Corporate Research" (CNR, 1994). After setting Italy's basic and industrial research in context, Bernardini reaches his conclusion about the mentality of the political and industrial leadership. Not one to mince words, he calls it "provincial". He could not have expressed it better. Italy is seen as ideologically reluctant to regard research and development as a collective necessity, at least as a safeguard against the international competitiveness of its commercial research products.

Hence, according to Bernardini, the need for something equivalent to the US Office of Technology Assessment, that is to say a centralized authority charged with the control, evaluation and planning of Italian research. His proposal goes



STONE ship carrying wine barrels, as depicted in *The Celtic World* edited by Miranda J. Green. This immensely detailed and comprehensive book will appeal to the informed general reader as well as the specialist. Routledge, £130.

beyond the US model, and his authority would command it a wide mandate. But although certainly attractive, the proposal is, alas, slightly too Utopian for today's Italy. For example, members of such a centralized body would be required to be truly independent of political power, in contrast to the current reality, where the system frequently puts in place "i consiglieri del Principe", chosen, of course, by the Prince himself.

Following Bernardini's assessment and proposals there is a discussion with several of the leading names in Italian academic and industrial research. Bruno Musso makes a strong case for technological research in industry, arguing that we need to relate management quality to research output quality. Ansaldo Ricerche is a case in point: from its heavy-duty mechanical experience with ships, trains and also nuclear-plant technology, this company has evolved the ability to manage those large-scale clockworks that are today's telescopes. And from experience with large electrical plants, it has also been able to provide the core parts of superconducting magnets for particle accelerators.

The discussion of basic research is more elaborate, left as it is to physicists of the calibre of Nicola Cabibbo, Luciano Maiani and Giorgio Careri. They touch on the lights and shadows of the great Italian research institutions, such as ENEA and INFN, and empathize with Bernardini's proposals. And Felice Ippolito puts in perspective the famous (or infamous, as the case may be) CNR "Progetti Finalizzati". We are shown how these national-scale research projects oriented towards one specific research objective are in fact increasingly evolving towards a lack of finalization, and the mixed quality of the

participation of the Italian academic world in them.

A hundred years ago, Guglielmo Marconi sent his famous first radio signal, opening a new era in communications. The Italian government of the time declined to support his work, feeling that communication through wire was perfectly adequate for every conceivable need. Marconi eventually offered his services to the British admiralty, which was obviously more interested. The question posed by Franco Praticco, a science journalist and writer, is: would any of the Italian governments of the following hundred years have behaved differently? (The answer is left as an exercise for the reader.) Accordingly, Praticco mentions a proposal that pays special attention to the vast, multidisciplinary research devoted to the restoration and maintenance of Italy's artistic treasures. In the same vein, Giorgio Ruffolo, a former minister for the environment, sees a dire need for an authority capable of defining and enforcing environmental research priorities in a country where "school is organized like that of Hammurabi in Babilonia". (While recognizing that this is a derogatory remark, I lack the necessary culture to judge whether it is aimed at Italian schools or Hammurabi. . .)

A small book, this pocket Laterza, but packed with new, substantial and well presented "idee per il governo" on science and research — and they come from a slate of scientists and industrialists thanks to whom Italy still commands international respect in many research fields. □

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Targeting of non-Ig sequences in place of the V segment by somatic hypermutation

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Affinity maturation of antibodies is characterized by localized hypermutation of the DNA around the V segment. Here we show, using mice containing single or multiple transgene constructs, that an immunoglobulin V_κ segment can be replaced by human β-globin or prokaryotic *neo* or *gpt* genes without affecting the rate of hypermutation; the V gene itself is not necessary for recruiting hypermutation. The ability to target hypermutation to heterologous genes *in vivo* could find more general applications in biology.

AFFINITY maturation of antibodies during the immune response is largely the result of hypermutation of the V segments and antigen-driven selection of mutants with improved binding properties¹. The hypermutation takes place in germinal centres, and occurs only during a narrow window of B-cell development²⁻⁶. Hypermutation introduces single nucleotide substitutions in a region of a kilobase or two that includes the integrated *V(D)J* of the immunoglobulin heavy and light chain genes^{7,8,11}; mutations are only rarely found in the immunoglobulin constant regions¹². The study of this unusual type of mutation has been greatly facilitated by the development of mouse lines carrying immunoglobulin transgenes which hypermutate at normal rates^{13,14}.

Although the V region itself is the physiological target of the hypermutation, it is not clear whether it is needed for full hypermutation or, indeed, whether non-immunoglobulin sequences can act as targets for the hypermutation machinery. A previous study¹⁵ found mutations in a *c-myc* allele that had been translocated next to the IgH locus in a Burkitt lymphoma; it was speculated that these mutations might have been introduced by the antibody hypermutation mechanism. So far, however, there has been no clear evidence as to whether somatic hypermutation can target heterologous sequences. In a variety of experiments with chimaeric transgenes, either the mutations observed were predominantly deletions and not single base substitutions as expected for somatic hypermutation¹⁶, or the mutation was either undetectable or only found at very low frequency such that they might not be ascribable to the hypermutation mechanism^{17,18}.

Here, using transgenes based on a construct known to be a good substrate for hypermutation¹⁴, we demonstrate that the hypermutation can effectively target a variety of non-immunoglobulin sequences that have been used to replace the V segment. Indeed, using a strategy in which a transgenic mouse line was created that carries multiple, distinctly modified transgenes, we show that the non-immunoglobulin sequences hypermutate as efficiently as and in a similar manner to their wild-type counterparts.

Arrays of modified transgenes

We have approached the problem using two types of transgenic mice. The conventional type contains a single modified, immunoglobulin construct in which the V segment is replaced by hetero-

logous sequence. In the second type we use a new strategy in that the mouse contains multiple, distinguishable transgenes. Not only does this mean that several transgenes can be tested at the same time, but also that one of the transgenes can be unmodified and serve as an internal control for mutation rates. The conventional type of animal carrying a single construct will expose possible *cis*-dominant effects from neighbouring transgenes.

All the transgenes used in this work are based on the rearranged immunoglobulin κ light-chain gene (*Lκ*) used previously¹⁴. The LκNG mouse line carries three modified forms: *Lκ-Vneo**Δ[XS]*i*, *Lκ-Vgpt**, and *LκΔB* (Fig. 1). In *Lκ-Vneo**Δ[XS]*i*, the bulk of the V segment (from FR2 to J_κ5) is replaced in frame by the bacterial *neo* gene but with a stop codon introduced into one of the complementarity-determining regions, CDR1. The chimaeric protein is therefore not produced¹⁹. To compensate for the increased distance between the V_κ promoter and the κ intron enhancer resulting from the *neo* substitution, *Lκ-Vneo**Δ[XS]*i* also has a deletion in the J_κ-C_κ intron. In *Lκ-Vgpt**, the V region extending from the ATG initiator codon through to CDR3 has been replaced by the bacterial *gpt* gene. The *gpt** gene in this construct has been mutated to render the protein enzymatically inactive. The third transgene construct in the LκNG mice, *LκΔB*, serves as the internal control and is closely related to the *LκΔ[3'FI]* gene used previously which mutates at normal rates²⁰.

In contrast to the LκNG mice, the Lκ-VβG line carries several (2–4) copies of a single transgene, which is a modified form of *Lκ* in which the V region (from the leader/FR1 border to J_κ5) has been substituted in-frame by a similar length of coding sequence derived from human β-globin complementary DNA.

Mutation rates of heterologous sequences

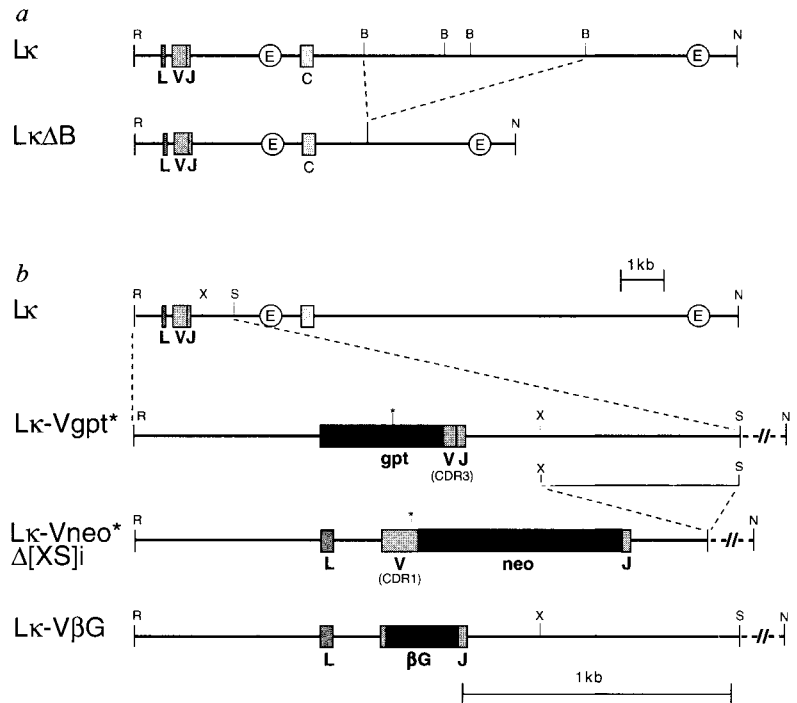
Two complementary approaches were used to analyse the hypermutation of the transgenes. In one, transgene sequences amplified by polymerase chain reaction (PCR) were cloned from a population of germinal-centre B cells from Peyer's patches. This shotgun approach²¹ allows the rapid accumulation of large databases. In the other, transgene sequences were determined from individually cloned hybridomas. This approach, although more laborious, eliminates PCR errors and allows comparison of hypermutations amongst individually marked components of the transgene array present in a single cell.

Peyer's patches were dissected from LκNG and Lκ-VβG mice and B cells fractionated by flow cytometry into PNA^{hi} (germinal centre cells which bind strongly to peanut agglutinin) and PNA^{low} populations, the latter serving as a control. Relevant DNA segments of the transgenes were amplified by PCR and

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FIG. 1 The transgenes. The constructs are modified versions of *L κ* (ref. 14). **a**, The immunoglobulin control transgene, *L $\kappa\Delta B$* , was derived from *L κ* by deletion of the DNA between the *Bam*HI sites 3' of *C κ* . **b**, The modified transgenes containing heterologous DNA. These are all analogous to *L κ* , differing only in the region between the *Eco*RI and *Sac*II sites, as shown in the enlargements. The deleterious amino-acid substitution *gpt* and the stop codon in *neo* are shown by asterisks. R, *Eco*RI; X, *Xba*I; S, *Sac*II; B, *Bam*HI; N, *Not*I.

METHODS. For *L $\kappa\Delta B$* , 5 kb of DNA 3' of *C κ* was removed from *L κ* by *Bam*HI partial digestion and religation. A modified version of *L $\kappa\Delta B$* (*L $\kappa\Delta B$ -7M*) was also created which differs only in that the *Pst*-*Kpn*I CDR1 fragment was replaced by a synthetic analogue containing seven silent mutations (not shown). For *L κ -Vgpt**, the *V κ Ox-1* gene segment from the ATG initiator codon (nucleotide position 1) to nucleotide 469 (Glu 79 of the κ chain) in at the FR3/CDR3 border was replaced by the entire 460-nucleotide coding sequence of *gpt*. Primers 5'-GCAGTAA-TTAGCTAGACACCAAAATTCAGCCACCATGGGCGAAAAAT-ACATCGTCACCTGGGAC-3' and 5'-CTGGCAGTAATAAGTG-GCAGCATCTTCAGCCTCTAGCGACCGGAGATTGGC-3' were used to amplify *gpt* from the pSV2gpt vector. The product was inserted by sticky feet mutagenesis⁴⁰ into an M13mp18 subclone containing the *V κ Ox-1/J κ 5* *Eco*RI-*Xba*I fragment. The resultant *Vgpt* *Eco*RI-*Xba*I fragment was then used to replace the equivalent fragment of *L κ* . The non-functional *L κ -Vgpt* gene (*L κ -Vgpt**) was obtained, following transfection of NSO myeloma cells, by *in vitro* selection for resistance to 6-thioguanine. In *gpt**, Val 86 of *gpt* (GTT) has mutated to Asp (GAT). *L κ -Vneo** has been described¹⁹. For *L κ -V β G*, a pUC18 clone containing the *Eco*RI-*Xba*I *V κ Ox-1* region but with *Sac*I and *Xho*I sites introduced at nucleotide 244 (codons 3/4 of the mature κ chain) and 547 (codons 104/105 of the mature κ chain) respectively of the *V κ Ox-1* exon was created by PCR using oligonucleotides 5'-GGAGACTGGGTGAGCTCAATTGTCTCTG-3' and 5'-TGGGACCA-AGCTCGAGATCAACGTAAGTA-3' on an *L κ* -derived template. The *V κ Ox-1* region between the *Sac*I and *Xho*I sites of the resulting plasmid was then replaced by a *Sac*I-*Xho*I human β -globin fragment generated by PCR amplification of β -globin cDNA with oligonucleotides 5'-CACCTGAC-



TCTTGAGCTCAAGTCTGCCGT-3' and 5'-CCATAGACTCACCTCGAGGT-CTCAGGATC-3', which introduce *Sac*I and *Xho*I sites into codon positions 7/8 and 104/105, respectively, of β -globin. The resultant *Eco*RI-*Xba*I fragment containing *V β G* was then used to replace the corresponding *Eco*RI-*Xba*I fragment of *L κ* . Transgenic mice were created by injecting vector-free DNA fragments into zygotes. For *L κ NG* mice, the injected fragments were an equimolar mixture of *Eco*RI-*Not*I fragments of *L $\kappa\Delta B$* , *L $\kappa\Delta B$ -7M*, *L κ -Vgpt**, and *L κ -Vneo**Δ[XS]i, whereas *L κ -V β G* was injected on its own. Founders were identified by expression of rat *C κ* in the serum or by Southern blot of tail DNA and bred with (C57BL/6x κ CBA) *F₁* mice. In the *L κ NG* line used in this work, *L $\kappa\Delta B$ -7M* contributed two of the five *L $\kappa\Delta B$* copies.

cloned into M13. Sequence analysis of the *gpt** region from 67 *L κ -Vgpt** clones revealed that 73% were mutated with 58% of the total containing more than one mutation (Fig. 2). Both the distribution of clones with respect to their degree of mutation as well as the average overall frequency of mutation (12.4×10^{-3} mutations per base pair (Table 1)) are very similar to those obtained with the internal control *L $\kappa\Delta B$* transgene (11.4×10^{-3} mutations per base pair (Table 1 and Fig. 2)). Not only are these mutation frequencies greatly in excess of what can be ascribed to PCR error, but they are also much higher than those detected in the *PNA^{low}* populations of B cells from the same transgenic lines (Table 1 and Fig. 2).

Mutation was also evident in the *L κ -Vneo**Δ[XS]i transgene, although to a lesser extent in that only 35% of the sequences were mutated, with 25% carrying more than one mutation. The lower overall mutation frequency deduced for this transgene (4.6×10^{-3} per base pair) reflects the increased proportion of unmutated clones rather than a decreased extent of mutation in those clones that are mutated (Table 1 and Fig. 2). We suspect that this difference between the *L κ -Vneo**Δ[XS]i and, say, *L κ -Vgpt**, transgenes is due to the fact that, whereas the *L κ -Vgpt** transgene is present in only a single copy, the *L κ -Vneo**Δ[XS]i transgene is present in four or five copies, with one or two of these copies being unable to mutate (see below).

The ability of the hypermutation mechanism to target heterologous sequences in place of the V segment is also evident in a different line of mice. In animals carrying only the *L κ -V β G* transgene, mutations accumulate in the human β -globin sequences at the same frequency and to the same extent as they do in the V gene segments of *L κ NG*; mutations are also found

to a much greater extent in the *PNA^{hi}* (germinal centre) than *PNA^{low}* cell populations (Table 1 and Fig. 2).

Hypermutation does not require the V

From these results, we can further restrict the parts of the κ gene required for optimum hypermutation. Although the leader intron includes the 5' border of the *V κ Ox-1* mutation domain²², it is notable that the *L κ -Vgpt** transgene mutates despite lacking this intron. Taken with results showing that the V gene promoter could be substituted²⁰, our findings that the coding sequences of the V segment can also be substituted by non-immunoglobulin DNA suggest that there are no sequences unique to the entire V gene segment necessary for recruiting hypermutation. Regarding the rest of the κ gene, the results with the *L $\kappa\Delta B$* transgene confirm that most of the sequence between *C κ* and the 3' enhancer is not required for efficient hypermutation. As far as the region between the V and C is concerned, although the κ -intron enhancer/matrix attachment region is essential²⁰, results with *L κ -Vneo**Δ[XS]i suggest that 776 nucleotides of DNA between the *Xba*I and *Sac*II sites close to the *J κ* cluster are dispensable, although we cannot exclude a possible compensating effect of neighbouring transgenes. This information should further simplify the construction of artificial mutation substrates.

Mutations in hybridomas

As an independent approach to monitoring hypermutation, we established secondary-response hybridomas from the spleens of *L κ NG* mice that had been immunized with 2-phenyl oxazolone conjugated to chicken serum albumin. Segments of the

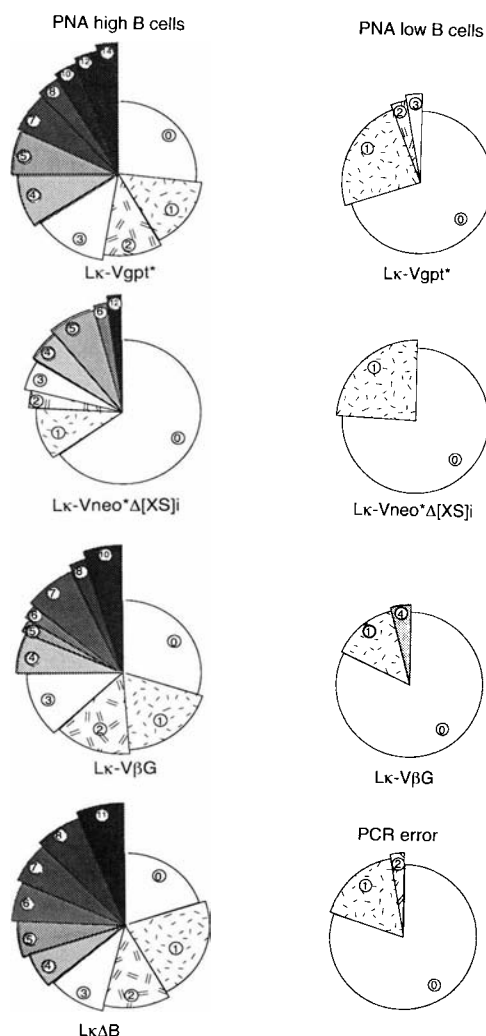


FIG. 2 Frequency distribution of clones with respect to the number of mutations they carry. Each part depicts the proportion of sequences with mutations as specified. The control for PCR error is described in the legend to Table 1.

*Lκ-Vgpt**, *Lκ-Vneo*Δ[XS]i* and *LκΔB* (control) transgenes were cloned by PCR from these hybridomas and possible PCR artefacts eliminated by sequencing multiple clones from each hybridoma. The *Lκ-Vgpt** transgene, which exists in single copy in the genome, is mutated in half the hybridomas that contain a mutated *LκΔB* control (Table 2). There are four copies of the *Lκ-Vneo*Δ[XS]i* transgene and, in some of the hybridomas, three of the copies are mutated. The fourth copy probably cannot mutate, as an unmutated PCR product is usually obtained, even from hybridomas in which all the other transgenes carry multiple mutations. Furthermore, Southern blot analysis (not shown) suggests that one or two of the *Lκ-Vneo*Δ[XS]i* copies may be truncated.

Although there is general agreement between the Peyer's patch and hybridoma results, in the latter the average number of mutations accumulated in the *LκΔB* transgene is slightly higher than in the other transgenes. This is probably due to antigen selection as it is observed in all but one of the hybridomas, this being the one where the *LκΔB* transgene copies do not provide the light chain of the hapten-specific antibody as shown by enzyme-linked immunosorbent assay (Table 2). Furthermore, in five of the hybridomas the *LκΔB* transgene mutations include amino-acid substitutions at His 34 or Tyr 36 that are characteristic of antibodies with increased affinity for 2-phenylloxazone^{23,24}.

Even within a single cell there is a considerable difference in the number of accumulated mutations in individual transgene copies (Table 2). This phenomenon cannot reflect a skewing effect of antigen selection because it is also observed in the case of *Lκ-Vgpt** and *Lκ-Vneo*Δ[XS]i*. Contrasting the mutations in, say, hybridomas C10.5.4 and C23.1.5, it is evident that the different extents of mutations are not inherent to the transgene construct itself; in one cell, one transgene accumulates more mutations, whereas in a second cell the other transgene does.

An unequal accumulation of mutations between transgene copies has previously been noted using selectable transgenes²⁵; we now confirm this finding for non-selectable ones, demonstrating that the skewing is not attributable to antigen selection. A model was proposed to explain these results²⁵ in which hypermutation is linked to the origin of replication. However, we think the explanation might be that, at least in some clones, when a gene is targeted the mutations are not introduced at an average of one per gene as usually assumed^{26,28}, but rather at a rate that allows the introduction of multiple mutations in a single round. The proposal is of a relatively low targeting efficiency of successive bursts of hypermutation exhibiting a higher rate than the calculated average of $4-10 \times 10^{-4}$ per base pair per generation^{23,26}.

Nature of the mutations

Studies of the intrinsic features of immunoglobulin hypermutation dissociated from the skewing effects of antigen selection have revealed that the process predominantly creates nucleotide substitutions (rather than deletions, insertions or inversions) with a bias in favour of transitions over transversions^{8,11,29,33}. These intrinsic properties of somatic hypermutation are retained when targeted to non-immunoglobulin sequences. Thus the vast majority of the mutations observed in the *Lκ-Vgpt**, *Lκ-Vneo*Δ[XS]i* and *Lκ-VβG* transgenes are nucleotide substitutions (Table 3). This contrasts with mutations of the *Lκ-Vneo*Δ[XS]i* gene when selected in myeloma transfectants (which do not hypermutate), where deletions are twice as common as point mutations³⁴. Like immunoglobulin gene substrates, mutations in the *Lκ-Vgpt**, *Lκ-Vneo*Δ[XS]i* and *Lκ-VβG*

TABLE 1 Transgene mutations in PCR clones derived from Peyer's patch B cells

Transgene	No. of clones†		Total mutations	Mutations per 10 ³ base pairs‡	
	Total	>1 mutation		Total	>1 mutation
B220 ⁺ PNA ^{hi} B cells					
<i>LκΔB</i>	50	29	161	11.4	18.3
<i>Lκ-Vgpt*</i>	67	39	217	12.4	20.3
<i>Lκ-Vneo*Δ[XS]i</i>	57	14	83	4.6	16.3
<i>Lκ-VβG</i>	57	29	141	8.9	16.1
B220 ⁺ PNA ^{low} B cells					
<i>Lκ-Vgpt*</i>	33	2	13	1.5	
<i>Lκ-Vneo*Δ[XS]i</i>	29	0	7	1.0	
<i>Lκ-VβG</i>	28	1	8	1.0	
PCR error				0.9	

Peyer's patches from five-month-old mice. B220⁺ PNA^{hi} and B220⁺ PNA^{low} B cells were fractionated by flow cytometry as described²¹. The PNA^{low} population is largely composed of non-hypermutated cells and provides a maximum level of background error²¹. Genomic DNA was amplified using the primers VκOxback and LκFor²³ for *LκΔB* and *Lκ-VβG*; VκOxback and NeogenBH (5'-GCCGGATCCGCTC-AGAAGAACTCGT-3'), which prime at the 3' end of the *neo* gene, for *Lκ-Vneo*Δ[XS]i* and 5'-PRIMAOXBACK (5'-CCCAGATTCTTAGGTGAGTATCTCTC-3') and Lygpt2 (5'-CGCGGATCCACGGCTGTTCAA-3'), which recognize the 5' region of V_HOx-1 and the 3' end of *gpt*, respectively, for *Lκ-Vgpt**. PCR products were digested with *EcoRI* and *BamHI*, purified by agarose gel electrophoresis, cloned into M13mp18, and sequenced with the Sequenase kit (USB). *LκΔB* clones were selected by hybridization with AFR3 oligonucleotide²¹. The background PCR error was estimated using transgenes from characterized hybridomas.

† Number of PCR-generated clones sequenced, and number of those sequenced that carry more than one mutation.

‡ Average number of mutations per 1,000 base pairs, either computed for all clones sequenced or only for those carrying two or more mutations.

TABLE 2 LκNG hybridomas

Hybridoma	Anti-phOx antibody†		No. of mutations per copy‡									
	Rat-κ	Mouse-κ	LκΔB§				Lκ-Vneo*Δ[XS]i				Lκ-Vgpt*	
C15.2.7	+	—	8	6	7	3	3	7	5	3	0	6
C10.5.4	+	—	8	5	6	1	1	9	7	3	0	1
A6.8	—	+	3	3	1	0		6	4	2	NF	3
C23.1.5	+	—	7	4	2	NF		2	0			8
D1.1.5	+	—	10	10	0			3	1	0		0
B20.6	+	—	4	4	2	0		2	0			0
C6.3.6	+	—	8	5	4	2	NF	0				0
D12.3.8	+	—	2	0				0				1
C4.6.8	+	—	2	0				0				1
D23.4	+	—	1	1	1	0		0				0

There are five copies of *LκΔB*, four or five of *Lκ-Vneo*Δ[XS]i*, and one of *Lκ-Vgpt** in the genome of *LκNG*. A four-month-old *LκNG* mouse was immunized with 2-phenyloxazone coupled to chicken serum albumin and hybridomas prepared 3 days after boosting as described³⁹. Culture supernatants were screened for anti-phOx antibodies, and positive wells cloned by the soft agar method³⁹. The three types of transgenic DNA were separately amplified, cloned and sequenced as described in Table 1. For each hybridoma, at least 5 (*Lκ-Vgpt**), 12 (*Lκ-Vneo*Δ[XS]i*) or 15 (*LκΔB*) clones of each type were sequenced. Mutants are defined by a minimum of two identical sequences.

† The nature of the light chain of the antibody was determined by ELISA, as described³⁸.

‡ Number of mutations found in each transgenic copy. When no mutations are found in several M13 clones, they may derive from more than one unmutated genomic copy. NF, the expected copy was not found among a minimum of 25 clones. Absent copies could result from instability of the transgenic DNA in B cells or in the derived hybridomas.

§ The results from the two versions of *LκΔB* copies (see Fig. 1 legend) have been pooled.

|| One of them is a single nucleotide deletion.

transgenes show a preference for transitions, about 60% (calculated from Table 3) rather than the 33% anticipated on a random basis. However, as shown below, some other features of hypermutation appear to be dependent on the nature of the DNA substrate.

Non-random distribution of mutations

The distribution of intrinsic mutations along an immunoglobulin V segment reveals that certain nucleotides are hotspots for mutation whereas others are rarely targeted³³. Hotspots are often concentrated in strategic segments. For example, in *VκOx-I*, many of the hotspots are found³⁰ in CDR1. In addition, the likelihood of each of the four nucleotide bases being targeted for mutation is not equal (this is often referred to as strand discrimination)^{8,11,32,33}. These features have been observed by

analysing mutations in *V* genes. The database of mutations in non-immunoglobulin targets allows us to go further and analyse the extent to which the observed intrinsic and non-random pattern of immunoglobulin mutation is affected by the nature of the immunoglobulin *V* gene sequence itself, given that this sequence will have evolved to function as a hypermutation substrate.

Hotspots. Clear hotspots exist in the distribution of mutations in the *Lκ-Vgpt**, *Lκ-Vneo*Δ[XS]i* and *Lκ-VβG* transgenes (Fig. 3). These hotspots are not at a conserved distance from the transcription start site, rather their positions seem more to be a consequence of local DNA sequence. The major hotspots in *Lκ-Vgpt** occur in the third base of Glu 70 and first base of Leu 71. The DNA sequence GAGCTT (underlined nucleotides are the hotspots) includes the triplet for the Ser codon AGY, but in an out-of-frame position. We believe that this is not simply coincidence, as we have previously reported that many of the hotspots in *V* segments occur in serine AGY codons^{30,33}. Some of the hotspots in the other transgenes are less dominant, but also conform well to the consensus sequences proposed as preferred sites for mutation in *V* segments^{33,35}. Hotspots in immunoglobulin genes do not always conform to such a simple rule (for example, sequences conforming to the hotspot consensus are by no means always hotspots in practice), and it is quite possible that features more subtle than simple linear motifs are important for localizing hypermutation to specific positions. This complexity may be less marked in hotspots of non-immunoglobulin targets which may therefore constitute a better substrate for refining the consensus.

R/S ratios. It is common to use the ratio of replacement/silent (*R/S*) mutations as a guide to antigen selection. In the case of *LκΔB*, the *R/S* ratio of the mutations observed (3.6) is identical to that predicted based on an assumption of a random mutation mechanism operating on the *VκOx-I* sequence. However, in the cases of transgenes carrying non-immunoglobulin sequences, the observed *R/S* values (3.8 for both *Lκ-Vneo*Δ[XS]i* and *Lκ-VβG*; 2.9 for *Lκ-Vgpt**) deviate from the predicted (3.4 in all three cases). This cannot be due to antigen selection but is likely to reflect the presence of intrinsic hotspots. Thus, for example, in the *gpt** sequences, many mutations are in the Glu 70 hotspot and are silent nucleotide transitions. Caution is therefore required in interpreting *R/S* ratios until the predicted values are calculated according to a rule that takes account of the non-random nature of somatic hypermutation.

Strand discrimination. The base substitutions in the *LκΔB* and *Lκ-Vneo*Δ[XS]i* transgenes show the same strand discrimina-

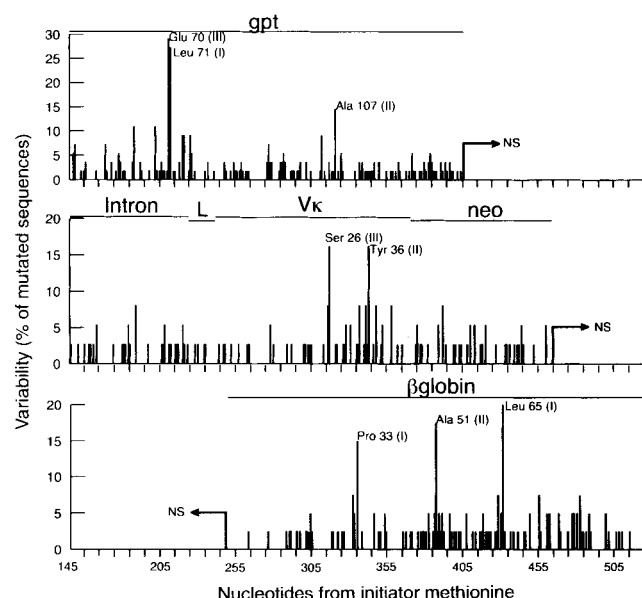
TABLE 3 Nucleotide substitutions

From	To				Total mutations (%)†
	T	C	A	G	
LκΔB					
T	—	0.48	0.44	0.08	25 (0.17)
C	0.77	—	0.08	0.15	39 (0.21)
A	0.31	0.19	—	0.50	54 (0.33)
G	0.07	0.21	0.72	—	43 (0.29)
Lκ-Vgpt*					
T	—	0.50	0.38	0.12	52 (0.21)
C	0.65	—	0.12	0.23	68 (0.31)
A	0.30	0.27	—	0.43	56 (0.25)
G	0.07	0.13	0.80	—	59 (0.23)
Lκ-Vneo*Δ[XS]i					
T	—	0.61	0.21	0.18	34 (0.22)
C	0.70	—	0.13	0.17	24 (0.17)
A	0.24	0.29	—	0.47	51 (0.40)
G	0.04	0.18	0.78	—	28 (0.21)
Lκ-VβG					
T	—	0.62	0.28	0.10	39 (0.28)
C	0.74	—	0.16	0.10	43 (0.31)
A	0.30	0.10	—	0.60	20 (0.19)
G	0.13	0.20	0.67	—	39 (0.22)

Data from Peyer's patch PNA^{hi} B cells. In *Lκ-Vgpt** and *Lκ-Vneo*Δ[XS]i*, the hybridoma data are also included.

† Number of mutations obtained in each of the four bases and, in parentheses, the proportion of mutations in each base but corrected for the base composition of the region analysed.

FIG. 3 Distribution of somatic mutations along the $L\kappa$ -Vgpt*, $L\kappa$ -Vneo* Δ [XS]i and $L\kappa$ -V β G transgenes. Variability at each nucleotide position is computed as the percentage of sequences carrying one or more mutations which are mutated at that position. The arrow (NS) indicates the limits of region sequenced. Nucleotides are numbered from the first base of the initiator ATG codon; the 5' border of mutation in $L\kappa$ is around nucleotide 145 (ref. 22). Major hotspots are indicated with the name and position of the amino acid followed by the position of the nucleotide in the codon (roman numeral).



tion identified in several immunoglobulin genes in that, on the coding strand, adenines (A) are more often mutated than thymines (T), and guanines (G) more often than cytosines (C)^{8,11,32,33}. The same bias is not revealed by the mutations in the *gpt** sequence and, indeed, an opposite bias is revealed by the $L\kappa$ -V β G transgene (Table 3). This suggests that the strand discrimination of hypermutation is, at least in part, dependent on the nature of the DNA substrate. This could occur if the likelihood of a base being mutated is dependent on the neighbouring sequence, as already indicated by the analysis of hotspots. In several models of hypermutation it is envisaged that strand discrimination is a consequence of mutation being initiated on a single DNA strand during replication, transcription or repair^{8,25,36,37}. Our results do not contradict these proposals, but demonstrate that at least part of the observed strand discrimination of hypermutation is imprinted in the immunoglobulin genes by their specific sequence.

Conclusions

Immunoglobulin light-chain genes are assembled by integrating one of the *V* genes into the *J-C* locus. However, the *V* gene fragment does not contribute any unique sequences required for

hypermutation. Thus it has been possible to study the hypermutation pattern of heterologous sequences. These non-immunoglobulin sequences (which are not evolutionarily adapted to serve as substrates for the hypermutation) still reveal the characteristic features of antibody hypermutation: a similar rate, a virtual absence of deletions or insertions, a preference for transitions over transversions, and typical hotspots. However, they also reveal interesting differences, such as an altered position of the hotspots with respect to the transcription start, altered ratios of replacement to silent mutations, and changes with respect to the preferential targeting of purines. Thus these features depend at least in part on the nature of the DNA substrate.

The analysis was performed using mice carrying multiple transgenes, one of which served as an internal control (particularly for mutation rate). Mice carrying transgene arrays could be useful in other fields of study. More importantly, the fact that the three target DNAs chosen at random (two of them bacterial) hypermutate when attached to the required controlling elements opens up the possibility of using the antibody hypermutation mechanism to generate (and possibly select) mutant non-immunoglobulin proteins *in vivo*. □

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Crystal structure of a complex between interferon- γ and its soluble high-affinity receptor

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The crystal structure of interferon- γ bound to the extracellular fragment of its high-affinity cell-surface receptor reveals the first view of a class-2 cytokine receptor–ligand complex. In the complex, one interferon- γ homodimer binds two receptor molecules. Unlike the class-1 growth hormone receptor complex, the two interferon- γ receptors do not interact with one another and are separated by 27 Å. Upon receptor binding, the flexible AB loop of interferon- γ undergoes a conformational change that includes the formation of a 3_{10} helix.

INTERFERON- γ (IFN- γ) is a dimeric glycoprotein produced by activated T cells and natural killer cells^{1,2}. Although originally isolated based on its antiviral activity, IFN- γ also displays powerful antiproliferative and immunomodulatory activities. These activities are essential for developing appropriate cellular defences against a variety of infectious agents. The first step in eliciting these responses is the specific high-affinity ($K_d = 10^{-10}$ M) interaction of IFN- γ with its cell-surface receptor (IFN- γ R α). However, cell signalling requires the IFN- γ /IFN- γ R α complex to interact with at least one of a family of additional species-specific receptors that convey different cellular responses^{3,4}. One of these receptors, identified as accessory factor 1 (IFN- γ R β 1), has recently been cloned and characterized^{5,6}. The IFN- γ -induced aggregation of the IFN- γ R α with the IFN- γ R β 1 or other receptors has recently been shown to induce the association and phosphorylation of two protein tyrosine kinases (Jak-1 and Jak-2), which in turn stimulate nuclear transcription activators that initiate the appropriate biological responses^{7,8}.

IFN- γ R α and IFN- γ R β 1 are members of the class-2 cytokine receptor family that also includes the cellular receptors for interferon- α and - β subtypes, interleukin-10, and the membrane tether for coagulation protease factor VII, tissue factor^{5,6,9–13}. Like their class-1 counterparts, they consist of an extracellular and cytoplasmic region connected by a single membrane-spanning peptide. The extracellular portions of the class-1 and class-2 receptors share a modular architecture consisting of a tandem set of fibronectin type-III (FBN-III) domains that are distinguished by unique cysteine sequence patterns¹⁰. Both classes of receptors start a signalling cascade by receptor oligomerization, which is initiated by ligand binding. Currently, crystal structure data on cytokine-receptor interactions are limited to the complexes of growth hormone with class-1 growth hormone and prolactin receptors^{14,15}.

Here we describe the 2.9 Å crystal structure of IFN- γ bound to the extracellular fragment of its high-affinity receptor. Common to the ligand binding surfaces of the class-2 IFN- γ and class-1 growth hormone receptors is the clustering of acidic or basic residues against exposed aromatic residues. These residue clusters are presented in a different geometric arrangement owing to the unique inter-domain angles of the two receptors. The orientation of the ligand on the receptor, which differs by 60° between IFN- γ and growth hormone, is critical for presentation of the initial complex for interaction with additional receptors. These results suggest a general pattern of recognition that may

be used by other cytokine-receptor family members. In addition, IFN- γ and growth hormone each use their flexible AB loops to facilitate receptor binding. This method of interaction may be used by other cytokines that display similar long-chain cytokine topology¹⁶.

Structure determination

The soluble extracellular fragment (sIFN- γ R α) of the human IFN- γ receptor containing an amino-terminal 8-residue deletion (residues 9 to 231) was expressed in *Escherichia coli*. The sIFN- γ R α binds IFN- γ with high affinity ($K_d = 10^{-9}$ M), based on competition and antiviral assays. A complex consisting of two sIFN- γ R α molecules and one homodimer of recombinant human IFN- γ (2 chains; residues 1 to 138) was purified by gel filtration and crystallized from 1.6 M phosphate solutions at pH 8.8. The crystals, which contain about 55% solvent, belong to the hexagonal space group $P6_322$ with cell dimensions of $a = 145.9$ Å and $c = 180.3$ Å. The crystallographic asymmetric unit contains one IFN- γ homodimer and two sIFN- γ R α molecules. The crystals diffract to approximately 2.9 Å when exposed to synchrotron radiation.

The structure of the interferon- γ receptor complex (IFN- γ RC) was solved by using a combination of molecular replacement and heavy-atom methods (Table 1 and Fig. 1). The R -factor for the structure is 22.3% for data between 8 and 2.9 Å. Several residues of IFN- γ (chain 1, residues 123 to 138, chain 2, residues 133 to 138) and the sIFN- γ R α (receptor 1, residues 230 to 231; receptor 2, residues 9 to 10 and 226 to 231) could not be modelled into the electron density maps. Crystal lattice interactions are more extensive for one receptor than the other, which correlates with their respective molecular mobilities. The average thermal parameters for the two receptors are 15 Å² and 49 Å², respectively.

Structure of IFN- γ

Each domain of the IFN- γ homodimer consists of six helices related by a non-crystallographic twofold axis. The two carboxy-terminal helices (E and F) from one chain associate with helices A, B, C and D from the twofold related chain (Fig. 2a). Helix F packs in a cleft created by helices A, B, C and D. Unique to helix F is a pronounced bend of 129° centred at Glu 112. With the exception of the AB loop, the helices are linked by short loops of 3 to 5 residues. The AB loop consists of 13 residues which encircle helix F. The main-chain after helix F

TABLE 1 Crystallographic statistics

Data set	Reflections				MIR analysis (15–3.5 Å)			
	Resolution (Å)	Measurements (no. of crystals)	Unique	Coverage (%)	R_{merge} (%) [*]	No. of sites	Mean isomorphous difference	Phasing power [†]
Native	2.9	114,915 (5)	23,530	91.7	6.80	—	—	—
K ₂ Pt(NO ₂) ₄	3.5	61,472 (5)	13,668	91.8	10.30	8	38.0	1.13
Pt(NH ₂ CH ₂ Cl) ₂	3.5	73,676 (4)	13,639	91.6	10.80	7	18.0	1.00
						r.m.s.		
Refinement	Resolution (Å)	Reflections $I > \sigma$	Atoms	R -factor (%)	Bond lengths (Å)	Bond angles (°)	B values (Å ²) [‡]	
	8–2.9	22,402	5,555	22.3	0.020	4.2	2.50	

Crystals of the purified IFN- γ RC (13 mg ml⁻¹) were obtained by vapour diffusion from 1.6 M ammonium phosphate solutions (pH 8.8) at 23 °C. Heavy-atom screening was done using an R-axis IIC image plate detector. Based on the low-resolution (6 Å) heavy-atom analysis, native and two derivative data sets were collected at the EMBL Hamburg outstation (DESY, $\lambda = 0.93$ Å). Data were integrated and scaled using the programs DENZO and SCALEPACK³⁸. Data manipulations were done in the CCP4 suite of programs³⁹. The position of IFN- γ in the cell was obtained by molecular replacement experiments, using an IFN- γ dimer as a probe, with the program Amore⁴⁰. Translation functions were calculated for the top 50 rotation function peaks in spacegroup P6₁22 and P6₅22. The highest correlation coefficient (c.c.) and lowest R -factor were obtained for the eighth rotation function peak in space group P6₅22 (15–4 Å data P6₅22: peak 1, $R = 44.1$, c.c. = 15.1; peak 2, $R = 46.1$, c.c. = 10.0; peak 1 in P6₁22, $R = 45.4$, c.c. = 10.4). Heavy-atom positions were obtained by difference Fourier and confirmed by analysis of the difference Patterson maps. Multiple isomorphous replacement (MIR) phases were calculated with MLPHARE⁴¹. The figure of merit for 13,998 reflections (15–3.5 Å) was 0.43. The anomalous signal was not used. MIR phases were combined with calculated phases using SIGMAA⁴². Interpretable electron density for one receptor was obtained from the combined phases modified by solvent leveling, histogram matching, and later, non-crystallographic symmetry (n.c.s.) averaging as implemented in the program DM (refs 43–45). A partial model for one receptor was built from polyalanine fragments from the GHR structure (3hrh; ref. 14; Protein data bank) with the graphics program Chain⁴⁶. Five rebuilding cycles consisting of positional refinement, phase combination, solvent levelling and map inspection resulted in an essentially complete model of one sIFN- γ R α . The second sIFN- γ R α was placed into position using the twofold matrix derived from IFN- γ and subjected to rigid body refinement. Inspection of the resulting phase-combined electron density map revealed a rotation of approximately 7° of domain 2 of the second receptor. Two additional rebuilding cycles, without density modification, allowed completion of the model which contains residues 9 to 229 and 11 to 225 for the two sIFN- γ R α s, and 1 to 122 and 1 to 132 for IFN- γ (poly-alanine for residues 125 to 132 to IFN- γ). The IFN- γ RC was refined by simulated annealing with the program X-PLOR⁴⁷. Domain 1 was constrained by the n.c.s. symmetry (residues 17 to 34, 44 to 54, 59 to 66, 70 to 90, 97 to 105). The r.m.s. deviation for residues not differing by crystal contacts is 0.19 Å for domain 1 and 1 Å for domain 2. The largest deviations for residues not influenced by crystal contacts occurs for receptor residues 134 to 142. Once the full atom model of the complex had been built and refined to an R -factor of 25.5%, tightly constrained B -factors were refined. The final R -factor for all structure factor amplitudes derived from intensity observations with $I > \sigma$ (8–2.9 Å, 22,402 reflections) is 22.3%. All non-glycine residues are in allowed regions of ϕ - ψ space. No solvent molecules have been included in the model.

^{*} $R_{\text{merge}} = \sum |I_h - \langle I_h \rangle| / \sum I_h$, where $\langle I_h \rangle$ is the average intensity over symmetry equivalents. Reflections with $I_h > \sigma$ were used in the calculations.

[†] Phasing power = $\sum |F_H| / \sum ||F_{\text{PHobs}}| - |F_{\text{PHcalc}}||$. Mean isomorphous difference = $\sum |F_{\text{PH}} - F_P| / \sum F_{\text{PH}}$ where F_{PH} and F_P are the derivative and native structure factor amplitudes, respectively.

[‡] r.m.s. deviation in B values is for all bonded atoms.

(residues 122 to 132) extends away from the molecule into the solvent.

The core structure of dimeric IFN- γ bound to the sIFN- γ R α generally agrees with the previously determined crystal structure of unbound human IFN- γ (ref. 17). The root mean square (r.m.s.) deviation between the bound and unbound dimers is 1.97 Å (246 Ca atoms). Removal of the N and C termini, the AB loop, and the CD loop from the calculation lowers the r.m.s. deviation to 0.78 Å (220 Ca atoms). The greatest structural differences occur in the AB loop (residues 18 to 26) where Ca atoms differ by as much as 7 Å. These large differences are consistent with the crystal and NMR analyses of unbound IFN- γ , which found the AB loop and C terminus to be flexible with no clear secondary structure^{17,18}. In contrast to unbound IFN- γ , owing to extensive interactions with the receptor, the AB loop is well ordered when IFN- γ binds to the sIFN- γ R α . Residues 20 to 23 in the centre of the loop form a well-defined turn of 3_{10} helix that runs perpendicular to helix F near the pronounced bend (Fig. 3b). The 3_{10} helix is not found in the AB loops of unbound IFN- γ . This strongly suggests that receptor binding induces the formation of the 3_{10} helix and ordering of the loop.

Structure of the sIFN- γ R α

The extracellular portion of the sIFN- γ R α folds into two domains to form a narrow rod-like molecule with overall dimensions of 75 Å × 25 Å × 15 Å (Fig. 2b). Domain 1 (D1, residues 17 to 105) and domain 2 (D2, residues 117 to 224) are connected by an 11-residue linker (residues 106 to 116). The inter-domain

angle between D1 and D2 is approximately 120°, which is similar to that described for tissue factor^{19,20}. Residues 9 to 16 at the N terminus and residues 225 to 229 (the stalk region) at the C terminus extend away from their respective domains.

Domain 1 of the sIFN- γ R α resembles an immunoglobulin fold with FBN-III domain topology²¹. The chain forms two β -sheets composed of β -strands A, B, E and G, F, C and C'. Several structures have been reported with equivalent topology, including domain 2 of CD2 and CD4, the chaperone protein pap D, tissue factor, growth hormone receptor (GHR) and prolactin receptor (PLR); however, the structure of D1 most closely matches D2 of the GHR^{14,15,19,20,22–25}. The r.m.s. deviation between D1 of the sIFN- γ R α and D2 of the GHR is 1.04 Å for 69 equivalent Ca atoms compared to 1.45 Å for 47 equivalent atoms in D1 of the GHR. Remarkably, the domain similarity extends to the main-chain conformation of the WSXWS equivalent sequence YGEFS (residues 222 to 226) observed in the GHR structure, as well as to the surrounding surface of alternating charged and aromatic residues^{10,14,15,26}. Tyr 99 from the WSXWS equivalent residues of the sIFN- γ R α (sequence ESAYA, residues 96 to 100) participates in the equivalent exposed surface located on β -strands C, F and G. The surface, which extends towards the CC' loop, is formed from residues Lys 89, Tyr 99, Arg 87, Trp 59 and Lys 50 for the sIFN- γ R α , and Arg 213, Phe 225, Arg 211, Trp 186 and Lys 179 for the GHR. A similar surface has also been observed in the PLR¹⁵. Although no function has been identified for this surface in any class-1 or class-2 receptor system, Lys 50 of the sIFN- γ R α forms a direct interaction with IFN- γ (Table 2 and Fig. 3b, c).

FIG. 1 Stereo view of representative electron density and refined model ($2F_o - F_c$ at 1.5σ) for receptor residues around the sequence TTEKS homologous to the D2 WSXWS box in the cytokine-receptor family. sIFN- γ R α residues 25–27, 112–115, 202–207 and 212–217 are shown. Note the interaction of Glu 215 with Tyr 26 from domain 1. All figures were produced with the program Ribbons⁴⁸.

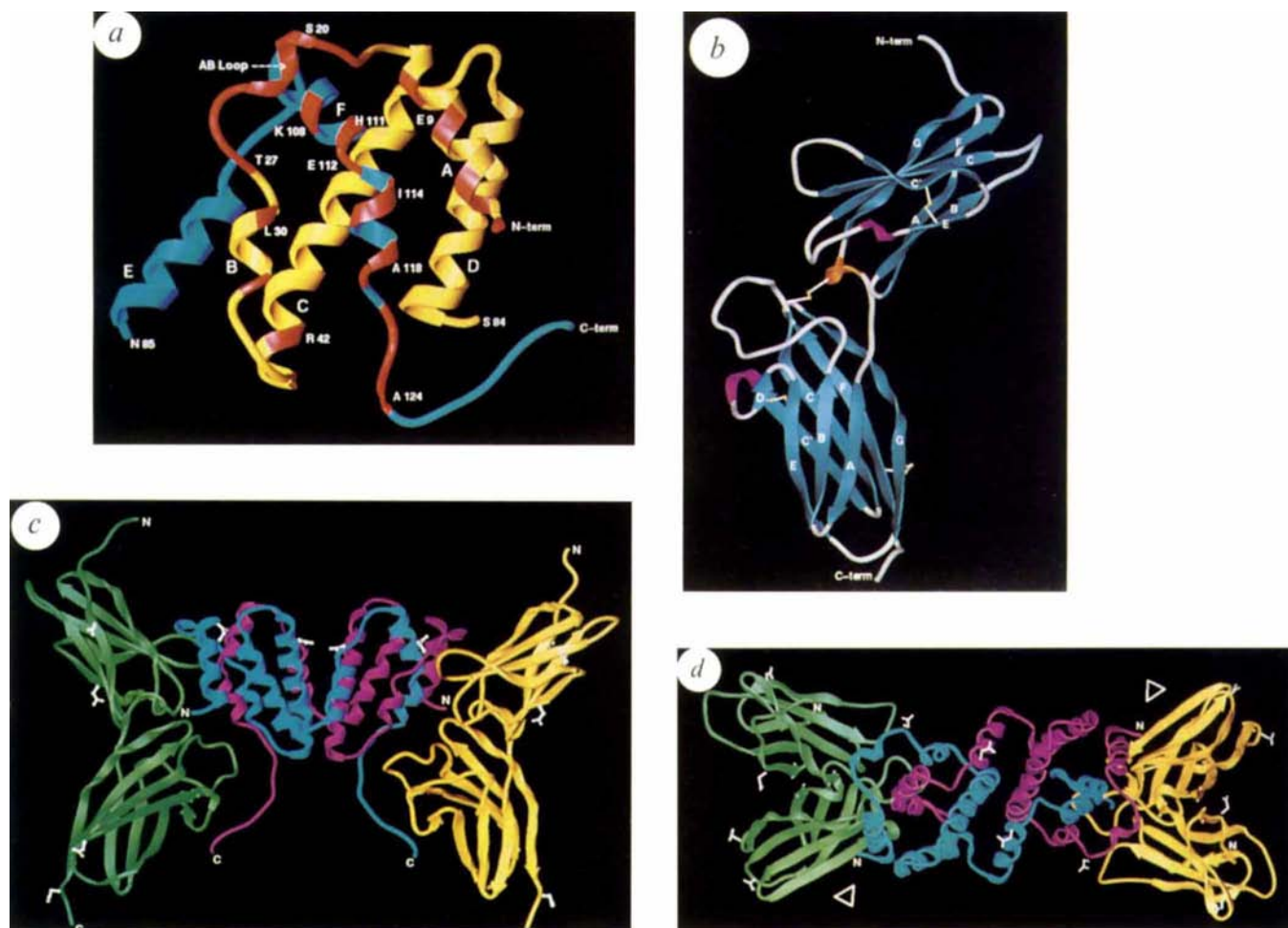
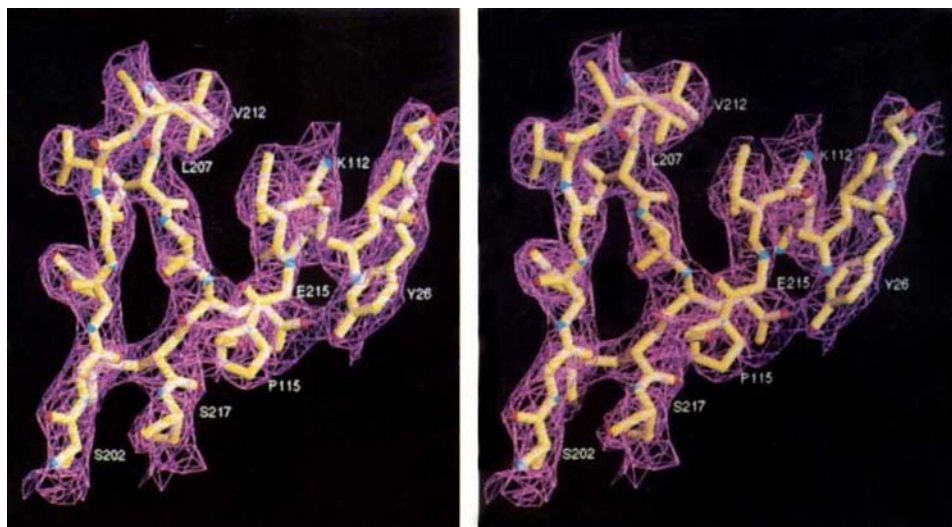


FIG. 2 Molecular structure of the IFN- γ RC and its components. *a*, Domain structure of IFN- γ . Residues 1–84 from chain 1 (yellow) and residues 85–132 (cyan) from chain 2. The helices are labelled from the N terminus A–F. Residues that contribute to the IFN- γ RC binding interface are shown in red. Selected residues are labelled. *b*, Secondary structure and disulphide bonds of the sIFN- γ R α . Secondary structure is colour-coded as follows: β -strands, blue; α -helix, orange; 3_{10} helix, magenta; loops, white. The C terminus of each β -strand is identified by an arrow. Residues that form the 7 β -strands of D1 are as follows: (A) 19–26, (B) 29–35, (C) 44–51, (C') 60–66, (E) 70–72, (F) 84–92 and (G) 95–105. The 8 β -strands for D2 are: (A) 117–123, (B) 126–132, (C)

154–164, (C') 167–174, (D) 179–182, (E) 185–192, (F) 198–207 and (G) 211–224. *c*, Structure of the IFN- γ RC with potential N-linked glycosylation sites. Each chain of IFN- γ and the sIFN γ R α is uniquely coloured. Residues 125–132 in IFN- γ are shown in the modelled position as described in the text and Fig. 3a. Residues 226–229 for receptor 2 (yellow) were generated by the n.c.s. matrix that relates domain 2 of the receptors. The view is perpendicular to the twofold axis of IFN- γ . The putative position of the cell membrane is located at the bottom. *d*, As *c*, but viewed parallel to the twofold axis of IFN- γ . The likely surface for interaction with the IFN- γ R β 1 is denoted with arrows.

Sequence comparisons of other class-2 cytokine receptors reveal only conservative substitutions for Tyr 99, Arg 87, and Lys 89. In contrast, residues Trp 59 and Lys 50 closest to the ligand binding site are not conserved.

The domain linker is defined by residues 106 to 116, which connect β -strand G in D1 to β -strand A in D2. Following β -strand G, residues 107 to 110 form a helical turn which has been observed in GHR, PLR and tissue factor^{14,15,19,20}. Cysteine 108 is

located on the helix and forms a disulphide bridge with Cys 153 located at the C-terminal end of the BC loop in D2. The disulphide bond, which is only observed in IFN- γ R α , is completely buried in the extensive domain interface. The total amount of surface area buried between the domains is approximately 1090 Å². The main-chain atoms of residues 111 to 114 participate in hydrogen bonds with main-chain and side-chain atoms donated from residues 25 to 27 on β -strand A in D1. Proline 116, which is conserved in all class-1 and class-2 members of the receptor family, forms no hydrogen bonds in the sIFN- γ R α or GHR structures. Proline 116, by breaking the β -sheet hydrogen bonding pattern, may provide conformational flexibility to the linker to allow D1 to adopt its proper orientation with respect to D2.

The topology of D2 is also similar to a FBN-III domain, with two β -sheets composed of β -strands A, B, E, D and G, F, C, C'. Relative to GHR, the BC and C'E connections are extended by 10 and 9 residues, respectively. The increased length of these segments results in shorter AB and CC' loops compared with D2 of the GHR. The result of the shorter AB loop in sIFN- γ R α is to remove residues that could form a receptor-receptor interaction (binding site 3) analogous to that observed in the GHR complex (Fig. 4). Domain 2 of the sIFN- γ R α contains two disulphide bonds. The first, which is unique to the human and murine IFN- γ R α 's, links Cys 186 located on β -strand E with Cys 181. The buried disulphide connects and supports a three-residue β -strand which is unique to FBN-III domains and forms hydrogen bonds with β -strand E. Following conventions for immunoglobulins, this β -strand is labelled D because it is part of the A, B, E, β -sheet²¹. With the exception of Cys 181, β -strand D and its adjacent residues (residues 178 to 183) display five solvent accessible glutamic and aspartic acid residues on the

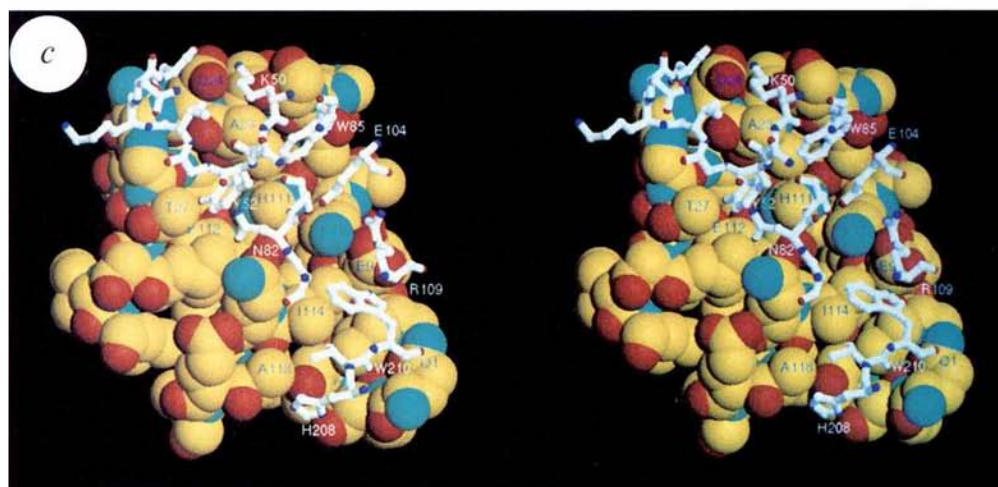
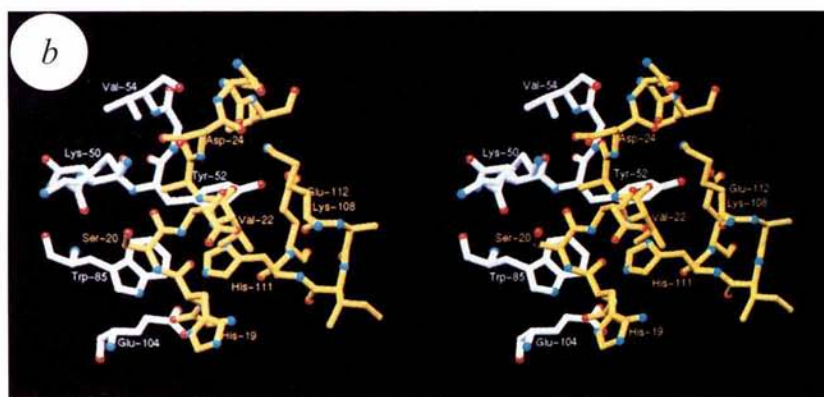
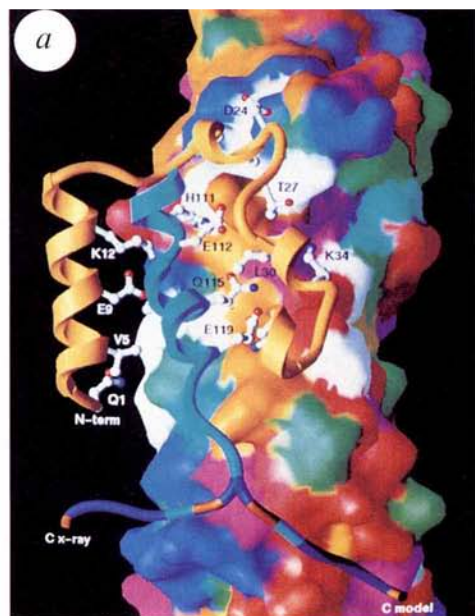


FIG. 3 The IFN- γ /sIFN- γ R α binding interface. **a**, The interface is viewed from the twofold axis of IFN- γ . Secondary structure and selected side chains are shown for IFN- γ residues 1–42 and 108–132 on the accessible surface of the sIFN- γ R α . Main-chain residues 1–42 and 108–124 are yellow and cyan, respectively. The surface of the receptor and residues 125–132 for IFN- γ are coloured according to amino-acid type using the following code: green, A, V, I, F, L; red, D, E; blue, R, K; white, W, Y, G, P; cyan, H; orange, S, T; yellow, C, M; purple, Q, N. The X-ray-determined and modelled positions of the IFN- γ C terminus are labelled. The model C terminus is located near the acidic patch on β -strand D. The surface was produced using the program ms-tri⁴⁹. **b**, Stereo view of the induced 3₁₀ helix in the AB loop of IFN- γ bound to the sIFN- γ R α . Carbon atoms are white on the receptor and yellow on IFN- γ . Nitrogen and oxygen atoms are cyan and red, respectively. **c**, Stereo view of the interface as viewed from the receptor. IFN- γ is represented as a space-filling model and the receptor by balls and sticks. For IFN- γ , carbon atoms are yellow, with oxygen red and nitrogen cyan. The sIFN- γ R α carbon atoms and bonds are white, with oxygen red and nitrogen purple. Selected residues are labelled.

FIG. 4 Stereo view of the IFN- γ and GHR (binding site 1) complexes. Domain 2 of each receptor was superimposed (r.m.s. deviation is 1.54 Å for 63 equivalent atoms within 2.5 Å of one another). Regions of IFN- γ (residues 20–24, 111–112) and GH (residues 61–67, 175–176) that make the most significant interactions with the receptor are shown in white. The receptor AB loop in D2 which forms most of the receptor–receptor interaction in the GHR complex is marked with an asterisk. The following colour code is used: sIFN- γ R α , yellow; GHR, pink; IFN- γ , cyan; and GH, red.



receptor surface. The BC loop contributes an additional five acidic residues that combine with β -strand D to form an acidic patch near the domain interface (Fig. 3a). Strands G and F are linked by an exposed disulphide bond (Cys 200–Cys 221) which is conserved in other class-2 receptors. Before Cys 221, the normal main-chain hydrogen-bonding pattern of the β -sheet is disrupted for residues 214 to 219. The O γ atoms of Thr 214 and Ser 217 form hydrogen bonds to the main-chain nitrogen atoms in β -strand F. In addition to these interactions, the O ϵ 2 atom of Glu 219 forms hydrogen bonds to the O γ of Ser 202, whereas the O ϵ 2 of Glu 215 forms hydrogen bonds to the OH of Tyr 26 on β -strand A in D1. Residues 213 to 217 (the amino-acid sequence is TTEKS) in this interaction correspond to the WSXWS box identified in most cytokine-receptor family sequences¹⁰ (Fig. 1). Thr 213 and Lys 216, corresponding to the tryptophan residues, are exposed to the surface as observed in the PLR structure, although the extended surface of alternating aromatic and charged residues is not conserved^{15,26}.

Structure of the IFN- γ /IFN- γ R α complex

Two sIFN- γ R α molecules bind the identical twofold related surfaces of the IFN- γ dimer to form a 2:1 sIFN- γ R α : IFN- γ complex (Fig. 2c, d). The overall dimensions of the complex are approximately 120 Å $\times$ 75 Å $\times$ 35 Å. The subunits of IFN- γ , as well as D1 from each receptor, are related by a non-crystallographic twofold axis, and D2 from one receptor can be superimposed on the other by a rotation of 173°. Domain 2 of each receptor makes an angle of about 60° with the probable position of the cell membrane. In this orientation, D1 assumes an angle complementary to the V-shaped IFN- γ dimer. As a result, the twofold axis of IFN- γ is perpendicular to the cell membrane, which allows an equivalent interaction to occur on its twofold related binding surface. While the stalk regions of each receptor are thought to be flexible, binding of the two receptors to IFN- γ would be expected to form a fairly rigid ternary complex. Although IFN- γ oligomerizes two IFN- γ R α s, they do not interact with one another and are separated by 27 Å at their closest point.

Residues of IFN- γ in the binding interface are located on two discontinuous polypeptide segments (residues 1 to 42 and 108 to 124). The first segment contains helix A, the AB loop, and helix B; the second contains helix F and the C terminus (Fig. 3a). Receptor residues are donated from the CC' and EF loops, the F and G β -strands of D1, the helix in the linker, and the BC and FG loops from D2. The BC and FG loops of D1 are analogous to the immunoglobulin hypervariable loops L1 and L3. In contrast to immunoglobulins, the binding segments on D2 are located on the C-terminal end of the domain, as observed in the GHR and PLR complexes. IFN- γ is oriented with its N and C termini near the D1D2 interface with the AB loop interacting with the CC' loop on D1. A total of 960 Å² of accessible surface

is buried in each binding interface. The aromatic receptor residues Tyr 52 (144 Å²), Trp 210 (95 Å²) and Trp 85 (75 Å²) contribute almost one-third of the total buried surface area. Tyr 52 and Trp 85 form a large protruding surface which makes extensive contacts with the AB loop, His 111 and Glu 112 of IFN- γ . Of the 11 hydrogen bonds and salt bridges identified in the interface, 5 involve main-chain atoms of the AB loop (Table 2 and Fig. 3b). On the opposite end of the molecule, IFN- γ residues Val 5, Ile 114 and Ala 118 pack against Trp 210 and Val 209 of the receptor (Fig. 3c). Differences between the human and murine IFN- γ sequences in these areas alone are sufficient to explain the species specificity of the human receptor for human IFN- γ .

Receptor binding assays using IFN- γ mutants and synthetic peptides have identified His 111, AB loop residues and the C terminus as critical for receptor binding^{27–32}. These results correlate well with the crystallographically determined binding interface, except in the C terminus. In the IFN- γ RC, the C-terminal IFN- γ residues 122 to 138 extend into the solvent and do not appear to be important for receptor binding. However, IFN- γ C-terminal deletion mutants, such as 1 to 129, show a sharp drop in receptor binding, but the 1 to 132 mutant does not³¹. This suggests that residues 130 to 132 (Lys–Arg–Ser) are critical for receptor binding. Consistent with the biochemical data, we suggest that the likely binding site for the basic tail of IFN- γ is the acidic cluster on the sIFN- γ R α ^{27,31,32}. However, in the crystal this interaction is prevented by a crystal contact which forces the C terminus to extend away from the acidic cluster towards the opposite surface of the receptor. A single rotation of 95° about Ala 124 places the C terminus in position to interact with the acidic cluster of the receptor (Fig. 3a). Although the modelled position explains the general requirement of IFN- γ for a basic charge at its C terminus for high-affinity binding, it cannot identify specific interactions made between the C terminus of IFN- γ and the sIFN- γ R α and possibly the IFN- γ R β 1.

TABLE 2 Hydrogen-bond and salt-bridge interactions in the IFN- γ RC

IFN- γ		sIFN- γ R α		Distance (Å)
Gln 1	N ϵ 2	Trp 210	O	3.0
Glu 9	O ϵ 1	Arg 109	NH2	2.9
Gly 18	O	Trp 85	N ϵ 1	2.9
Ala 23	O	Gly 53	N	3.2
Ala 23	O	Val 54	N	3.3
Asp 24	O δ 1	Lys 50	N ζ	2.9
Asn 25	N	Val 54	O	3.0
Gly 26	N	Val 54	O	2.8
His 111	N ϵ 2	Glu 104	O ϵ 1	2.9
Glu 112	O ϵ 1	Tyr 52	OH	2.8
Gln 115	N ϵ 2	Asn 82	O δ 1	3.3

Residues with atoms within 3.4 Å of each other are listed.

Glycosylation

The sIFN- γ R α contains five potential sites for N-linked glycosylation (Asn 20, 65, 72, 165 and 226). All five asparagines are fully exposed to the solvent and occur on three separate surfaces of the receptor. Asn 20 is located on β -strand A in D1. On the opposite edge of the β -sheet, Asn 65 and Asn 72 are located on β -strands C' and E. The last two sites (Asn 165 and Asn 226) lie at the base of D2 near the putative position of the cell membrane. If Asn 226 is glycosylated (it is not when expressed on insect cells³³), then it and Asn 165 could interact with the cell membrane and play a role in orienting the receptor prior to IFN- γ binding. In the IFN- γ RC, the two glycosylation sites on IFN- γ (Asn 25 and Asn 97) (ref. 34), which are adjacent to one another on the AB loop and helix E, extend the glycosylated surface formed by Asn 65 and Asn 72 (Fig. 2c, d).

Comparison of IFN- γ and GHR complexes

The main structural difference between the IFN- γ R α and GHR is the relative orientation of their domains. When D2 of one receptor is superimposed on D2 of the other, the orientation of the D1 domains differs by a rotation of 40° and a 7 Å translation (Fig. 4). Functionally, this structural difference allows the GHR to expose an additional hormone binding loop (the AB loop from D1). In contrast, the AB loops in IFN- γ R α and tissue factor are buried in their domain interfaces. The orientation of IFN- γ and growth hormone on their receptors differ by approximately 60° with IFN- γ assuming a more upright position. Comparison of the IFN- γ and growth-hormone binding surfaces presented by the receptors underscores the importance of aromatic residues (Trp and Tyr) in the binding interfaces. Even more striking is the conservation of charged residues (Arg, Glu or Asp) which pack against each tryptophan. Examples of these pairs are Trp 85/Glu 104 and Trp 210/Arg 109 in the IFN- γ R α , and Trp 104/Asp 126 and Trp 169/Arg 43 in the GHR. In the GHR complex, these two pairs of residues are responsible for more than three-quarters of the binding free energy³⁵. Although

IFN- γ and growth hormone use topologically equivalent helices and loops to form their receptor binding sites, the most significant interactions occur on opposite ends of the molecule, specifically, the AB loop for IFN- γ and the C-terminal end of the AB loop in growth hormone (residues 61 to 67). A cleft formed by the overhang of a small helix on the AB loop and the C-terminal helix of the cytokine (Fig. 4) is conserved in both interactions.

Putative binding site for IFN- γ R β 1

The biologically active IFN- γ receptor complex requires the species-specific interaction of the IFN- γ R α with at least one additional accessory factor, such as the IFN- γ R β 1 (refs 4, 36). Recent studies with a covalently linked IFN- γ mutant suggest that each domain of IFN- γ may function independently to transduce a biological signal³⁷. Structurally this corresponds to each domain of IFN- γ binding one IFN- γ R α and one IFN- γ R β 1. The potential interaction of different combinations of these accessory factors with the IFN- γ RC suggests an additional mechanism for IFN- γ to modulate its pleiotropic activities.

Where would the IFN- γ R β 1 bind to the IFN- γ RC? Based on antiviral assays with IFN- γ deletion mutants and receptor chimaeras the IFN- γ R β 1 interacts with the IFN- γ R α and with the C terminus of IFN- γ (refs 27, 31, 36). Comparison of IFN- γ and growth hormone bound to their receptors (Fig. 4) reveals a striking correspondence of the C terminus of IFN- γ with the N-terminal residues of growth hormone that form a major portion of the second GHR binding site. While the receptor-receptor interaction sites used by the GHR are not conserved in the sIFN- γ R α , the second GHR may be positioned with no bad contacts into the IFN- γ RC. In this model, the interface of the second GHR interacts with the C terminus of IFN- γ . The occurrence of different C termini in natural IFN- γ may allow alteration of the second binding site, which could provide an additional control mechanism of IFN- γ signalling. Further mutagenesis and crystallographic studies are necessary to validate this model. □

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Stochastic resonance without tuning

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STOCHASTIC resonance¹⁻⁴ (SR) is a phenomenon wherein the response of a nonlinear system to a weak periodic input signal is optimized by the presence of a particular, non-zero level of noise⁵⁻⁷. SR has been proposed as a means for improving signal detection in a wide variety of systems, including superconducting quantum interference devices⁸, and may be used in some natural systems such as sensory neurons⁹⁻¹⁵. But for SR to be effective in a single-unit system (such as a sensory neuron or a single ion channel), the optimal intensity of the noise must be adjusted as the nature of the signal to be detected changes¹⁵. This has been thought to impose a limitation on the practical and natural uses of SR. Here we show that the ability of a summing network of excitable units to detect a range of weak (sub-threshold) signals (either periodic or aperiodic) can be optimized by a fixed level of noise, irrespective of the nature of the input signal. We also show that this noise does not significantly degrade the ability of the network to detect supra-threshold signals. Thus, large nonlinear networks do not suffer from the limitations of SR in single units, and might be able to use a single noise level, such as that provided by the intrinsic noise of the individual components, to enhance the system's sensitivity to weak inputs. This suggests a functional role for neuronal noise^{14,16-18} in sensory systems.

We considered a summing network (Fig. 1) of identical excitable units, which were taken to be FitzHugh–Nagumo (FHN) model neurons (for details, see Fig. 1 legend). Each unit was subjected to a common input signal and its own independent noise source (which could be intrinsic or externally added to each unit). We assumed that information was transmitted by each unit via temporal changes in its firing rate. (This is a valid assumption for many types of sensory neurons¹⁹.) The network operated by summing the mean firing rate (MFR) signal from each unit to obtain a resultant MFR signal for the entire system (Fig. 1). The motivation for studying this system was twofold: (1) networks of this sort can be easily constructed and incorporated into signal-detection systems²⁰, and (2) such networks are appropriate models for many neurophysiological sensory systems²¹.

Recently, it was shown that SR-type behaviour can be characterized in excitable systems with aperiodic inputs²². This general type of behaviour was termed aperiodic stochastic resonance (ASR). To characterize ASR in the summing network of Fig. 1, we considered the normalized power norm C_3 given by

$$C_3 = \frac{S(t)R_\Sigma(t)}{[S^2(t)]^{1/2}[(R_\Sigma(t) - \bar{R}_\Sigma(t))^2]^{1/2}} \quad (1)$$

where $S(t)$ is an aperiodic (zero-mean) input signal (see Fig. 1 legend), $R_\Sigma(t)$ is the resultant MFR signal for the network, and the overbar denotes an average over time. (For ASR, the exact form of $S(t)$ is unimportant (it could be periodic, for example), provided that its variations occur on a timescale which is slower than the characteristic time(s) of the system under study²².) Maximizing C_3 corresponds to maximizing the coherence between the input stimulus $S(t)$ and the system response $R_\Sigma(t)$.

The numerical results for N -unit summing networks with a sub-threshold aperiodic input signal are given in Fig. 2. Shown are the values of C_3 as a function of the input noise intensity D . (The solid curves are from the theory to be described below.) The

results for a single-unit ($N=1$) network showed characteristic signatures of SR-type behaviour: a rapid rise to a clear peak, and then a slow decrease for higher values of D . As the number of units in the network was increased, the peak C_3 value increased, that is, it asymptotically approached 1.0 (Fig. 2). Thus, the stimulus-response coherence was enhanced as the size of the network was increased. This finding is consistent with those of other studies which have considered conventional SR in uncoupled²⁰ and coupled²³⁻³⁰ networks of nonlinear elements. In addition, and perhaps more importantly, as the size of the network was increased, the post-peak decay rate of C_3 (relative to the ASR peak amplitude) diminished, that is, C_3 asymptotically approached a plateau near 1.0 for a range of noise intensity values larger than the optimal value for a single unit (Fig. 2). This plateau indicates that for large N , maximum fidelity is attained for a wide range of noise values.

We have developed a theory²² of ASR for a single excitable unit, based on the following dynamics of the FHN model. (Kiss *et al.*^{31,32} independently developed a theory for SR-type behaviour in threshold devices with wideband input signals.) For a sub-threshold activation signal (see Fig. 1 legend), the FHN model has a stable fixed point. Input gaussian noise 'kicks' the system away from the fixed point. If the system is kicked over its threshold, it 'fires' and subsequently returns to the stable fixed point after a refractory period. To calculate the mean firing rate $R_i(t)$, which is proportional to the probability of escaping from the stable fixed point, we formulated the problem as a barrier-escape problem. We then used Kramers' formula for the escape rate³³ to derive the following expression for $R_i(t)$:

$$\langle R_i(t) \rangle \propto \exp(-\sqrt{3}[B^3 - 3B^2 S(t)]\varepsilon/D) \quad (2)$$

where the angle brackets $\langle \rangle$ denote an ensemble average, B is the signal-to-threshold distance (that is, the barrier height), and ε is an FHN model parameter (see Fig. 1 legend).

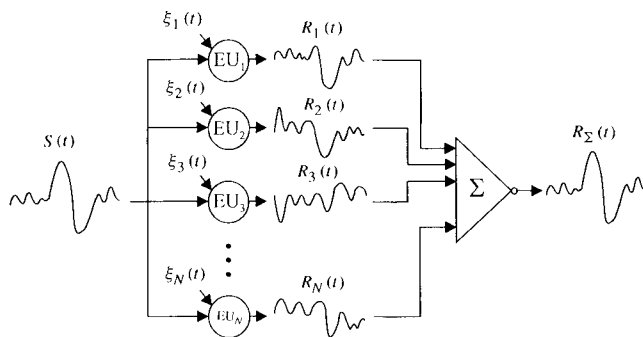


FIG. 1 A summing network of N excitable units (EU_i). Each EU_i was a FitzHugh–Nagumo model neuron¹³, governed by the following equations:

$$\begin{aligned} \dot{v}_i &= v_i(v_i - a)(1 - v_i) - w_i + A + S(t) + \xi_i(t), \\ \dot{w}_i &= v_i - w_i - b \end{aligned}$$

where $v_i(t)$ is a fast (voltage) variable, $w_i(t)$ is a slow (recovery) variable, A is a constant (tonic) activation signal, $\varepsilon=0.005$, $a=0.5$, $b=0.15$, $\xi_i(t)$ is unit-independent gaussian white noise with zero mean and autocorrelation $\langle \xi_i(t)\xi_j(t) \rangle = 2D\delta(t-t')$, the angle brackets $\langle \rangle$ denote an ensemble average over the noise distribution, D is the noise intensity, and $S(t)$ is a slowly varying aperiodic signal which was common to all units in the network. The time-varying mean firing rate (MFR) signal $R_i(t)$ for each unit, defined as the number of spikes per second produced by each unit, was computed by passing a unit-area symmetric Hanning window filter³⁴ over an impulse train corresponding to the firings of each unit, which were determined using the method described in ref. 13. The summer (Σ) operated by averaging the MFR signals from the respective units to obtain a resultant MFR signal $R_\Sigma(t)$ for the network. Each unit's MFR signal was equally weighted by the summer. (In ref. 20, conventional SR was examined in a similar network of Schmitt triggers.)

This analysis can be extended to a summing network of N excitable units. To derive an expression for C_3 , we need an expression for $R_{\Sigma}(t)$. We use the supposition that $R_i(t)$ is equal to the ensemble average (given by equation (2)) plus a fluctuating term which arises from the input noise. It can then be shown that $R_{\Sigma}(t)$ is equal to the same ensemble average plus a stochastic term which scales as $N^{-1/2}$. This rate expression can be substituted into equation (1) and with some algebraic manipulations and averaging operations, it can be shown that

$$C_3 = \frac{\Delta[\overline{S^2(t)}]^{1/2}}{[\exp(\Delta^2 \overline{S^2(t)}) - 1 + (\sigma^2(D)/N) \exp(2V/D) \exp(-\Delta^2 \overline{S^2(t)})]^{1/2}} \quad (3)$$

where $\Delta = 3\sqrt{3}\varepsilon B^2/D$, $V = \sqrt{3}\varepsilon B^3$ and $\sigma^2(D)$ is an empirical function of D (ref. 22). From equation (3), it can be seen that the theory matches the numerical data (Fig. 2), predicting that as N increases, C_3 asymptotically approaches a plateau near 1.0 for a range of noise intensity values larger than the optimal value for a single unit. (Note that it can be shown that equation (3) is unchanged for a summing network of non-identical excitable units, that is, ones with slightly different thresholds. The variation in B across the units of the network enhances the amplitude of $R_{\Sigma}(t)$ by a constant factor, which is normalized out in the calculation of C_3 (equation (1)).)

It can be shown from equation (3) that

$$N = \frac{aC_3^2}{1 - C_3^2} \quad (4)$$

where $a = \sigma^2[(1 - \Delta^2 \overline{S^2(t)})/\Delta^2 \overline{S^2(t)}] \exp(2V/D)$. Thus, for any D and σ^2 , one can calculate the number of network units needed to obtain a desired level of fidelity.

The plateau in the $C_3(D)$ curve (Fig. 2) of a relatively large (for example, $N = 1,000$) summing network suggests that a fixed

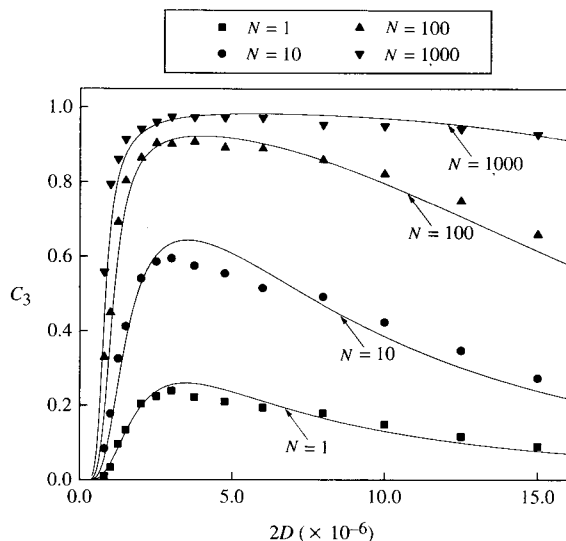


FIG. 2 Values (symbols) of the normalized power norm C_3 plotted against $2D$, where D is the intensity of the input gaussian white noise on each unit, for N -unit summing networks with a sub-threshold aperiodic input signal $S(t)$. The theoretical predictions (solid curves) from equation (3) are also shown. The model equations for each unit (see Fig. 1 legend) were solved numerically using an algorithm developed for stochastic differential equations³⁵. An integration stepsize of 0.001 s was used. The reported results did not change for smaller stepsizes. $S(t)$ was formed by convolving gaussian correlated noise (with correlation time of 20 s) with the filter described in Fig. 1 legend. The same input signal $S(t)$, with variance of 1.5×10^{-5} and total time length of 300 s, was used for all results presented. The signal-to-threshold distance B was 0.07.

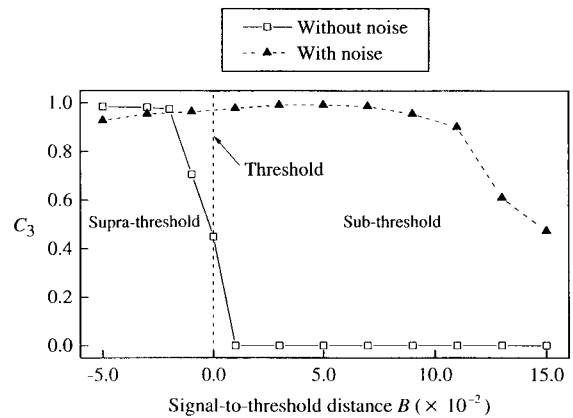


FIG. 3 Values of the normalized power norm C_3 versus signal-to-threshold distance B for a 1,000-unit summing network without and with fixed levels of independent noise on each of its excitable units. In the latter case, the intensity D of each unit's input noise was equal to 1.88×10^{-6} . This value was taken from the plateau region in the results for the 1,000-unit summing network in Fig. 2. The values for C_3 were determined using the methods described in Fig. 2 legend. The input signal $S(t)$ used in Fig. 2 was used for all results presented.

level of independent noise on each unit of such a network can optimally enhance the network's ability to detect a range of sub-threshold signals. This is in contrast to ASR²² (or SR¹⁵) in a single excitable unit, which requires the input noise intensity to be systematically modulated as a function of the changing characteristics (for example, variance or signal-to-threshold distance) of the signal to be detected. Importantly, the presence of a fixed level of unit-independent noise also does not significantly affect the ability of a relatively large summing network to detect supra-threshold signals (Fig. 3). With both supra- and sub-threshold signals, the averaging action of the network cancels out the incoherent errors introduced by the noise on each unit. Thus, additive noise reduces the 'effective threshold' of the network (Fig. 3), without significantly degrading the network's ability to detect supra-threshold signals. In this manner, the noise serves to extend the operating range of the overall system.

From a technological standpoint, these novel results suggest that additive noise could be incorporated into the design of a multi-component signal-detection system so as to improve the system's ability to detect weak signals. In addition, this work suggests that the noise intrinsic to each component, which has previously been viewed as a limiting factor for system performance, could be used to improve the sensitivity of the overall system.

Likewise, from a neurophysiological standpoint, these findings suggest a possible functional role for neuronal noise^{14,16,18} in sensory systems. It has been speculated that sensory systems may have evolved the capability to take advantage of SR^{5,10,12}. However, physiological mechanisms by which the noise on a given sensory neuron could be dynamically modulated as a function of the changing nature of a weak signal (one which would first have to be detected by some component of the nervous system) have not been identified. The present findings indicate that such mechanisms may be unnecessary, and instead constant levels of internal or external noise on each neuron in a sensory system could optimally enhance the overall response of the system to a range of sub-threshold signals, even for conditions wherein the individual responses of the neurons are not optimized. □

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Why gold is the noblest of all the metals

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THE unique role that gold plays in society is to a large extent related to the fact that it is the most noble of all metals: it is the least reactive metal towards atoms or molecules at the interface with a gas or a liquid. The inertness of gold does not reflect a general inability to form chemical bonds, however—gold forms very stable alloys with many other metals. To understand the nobleness of gold, we have studied a simple surface reaction, the dissociation of H_2 on the surface of gold and of three other metals (copper, nickel and platinum) that lie close to it in the periodic table. We present self-consistent density-functional calculations of the activation barriers and chemisorption energies which clearly illustrate that nobleness is related to two factors: the degree of filling of the antibonding states on adsorption, and the degree of orbital overlap with the adsorbate. These two factors, which determine both the strength of the adsorbate–metal interaction and the energy barrier for dissociation, operate together to the maximal detriment of adsorbate binding and subsequent reactivity on gold.

In discussing nobleness, we distinguish between the ability of the metal to form and break bonds at the surface, and its ability to form new compounds (such as oxides and carbides) or be dissolved. The latter ability is closely related to the former: to form a compound it is necessary that the metal is able to bond to other atoms, but the formation of a new compound or the dissolution of the metal also involves the breaking of the metal–

metal bonds. We first consider the surface reactivity of the metals and then comment on the consequences for the more general ‘bulk-nobleness’.

Figure 1 summarizes the calculated reaction energetics for H_2 on the (111) surface of the four metals, Ni, Cu, Pt and Au. It can be seen that H_2 dissociation is activated on Cu and Au, and non-activated on Ni and Pt, in complete agreement with experimental evidence^{1,8} and previous calculations where available (Ni, Cu)^{9–13}. It is also seen that Au stands out as having both the highest barrier for dissociation and the least stable chemisorption state. The lack of reactivity of gold is thus clearly borne out by the calculations.

As a starting point for analysing the repulsive hydrogen–gold interaction we consider in Fig. 2a the simple two-level interaction problem. When the electronic states of two atoms overlap, quantum mechanics dictates that they be orthogonal to each other. This drives up the energy and leads to so-called Pauli repulsion. However, the overlapping states will also hybridize and form bonding and antibonding states. If only the bonding state becomes occupied, the hybridization effect will be attractive and will counteract the orthogonalization energy cost. If, on the other hand, both the bonding and antibonding state become occupied no hybridization energy is gained and the orthogonalization energy cost prevails.

This simple two-level problem can be transferred to the case of hydrogen chemisorption on the transition-metal and noble-metal surfaces. Here the adsorbate–surface interaction is most conveniently considered in two steps^{14–16}. First, the interaction of the hydrogen 1s state with the metal 4s (Ni and Cu) or 6s (Pt and Au) band leads to a deep-lying filled bonding state and an empty antibonding state. This interaction is therefore attractive and, as the s–s coupling matrix element varies little for the metals considered, the attraction should be about the same for all four surfaces^{17,18}. Then follows the interaction of the bonding state with the metal d states. As illustrated in Fig. 2b this results in an extra bonding shift of the hydrogen-induced state, but also

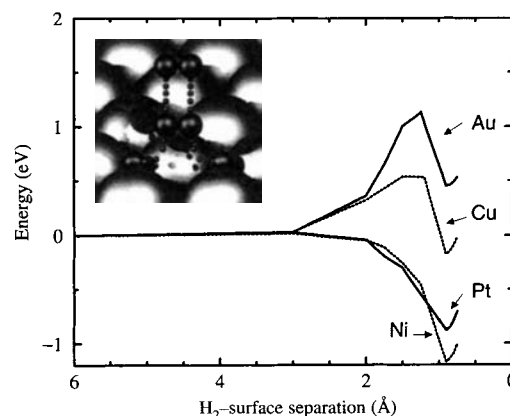


FIG. 1 Main figure, the calculated energy along the minimum-energy reaction path for H_2 dissociating on the (111) surface of Ni, Cu, Pt and Au. The H_2 –surface separation is the height of the molecular centre of mass above the plane of the surface atoms. For each height, the H–H bond length has been allowed to relax to give the lowest-energy configuration, and in a similar way the configuration of the molecule relative to the surface unit cell has been allowed to relax on the way towards the surface. Inset, the calculated reaction path for H_2 dissociating on the Au(111) surface. The reaction paths on the other metals also involve an orientation of the molecular axis parallel to the surface. The calculations are done for a super-cell geometry with one H_2 molecule per $\sqrt{3} \times \sqrt{3}$ surface unit cell on one side of metal slabs consisting of four atomic (111) layers. The total energies are calculated from density-functional theory²⁰ describing exchange and correlation effects in the local density approximation augmented by non-local corrections in the so-called generalized gradient approximation (GGA)²¹.

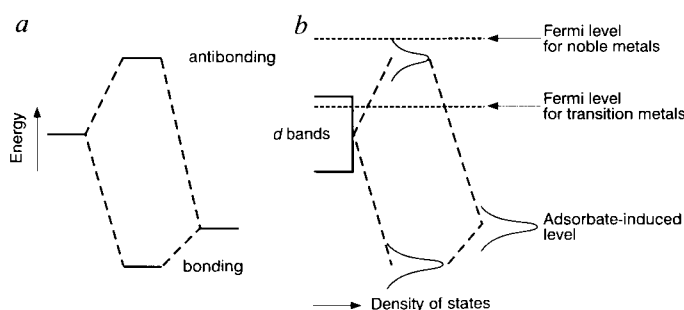


FIG. 2 Schematic illustration of the interaction between two electronic states. The down-shift of the bonding state is smaller than the up-shift of the antibonding state because the overlap of the initial states gives rise to an energy cost related to the orthogonalization of the two states. Both the energy associated with the orthogonalization, and the hybridization energy associated with the formation of bonding and antibonding states, scale with the square of the coupling matrix element. *a*, The simple case of two sharp atomic or molecular states. *b*, The interaction between a state of an adsorbate outside a metal surface, which has been broadened out to a resonance owing to the interaction with the metal *s* band, and the metal *d* bands.

in an antibonding state right above the metal *d* bands. The energy associated with this hybridization interaction must depend on the filling of the antibonding state and the coupling matrix elements V_{sd} between the *s* state and the *d* states, and we suggest that it is this contribution to the bonding which determines all the trends in the atomic chemisorption energies seen in Fig. 1.

To see this, we consider now the self consistently calculated density of one-electron states (DOS) of chemisorbed hydrogen on the four surfaces (Fig. 3). The results of the full calculations are seen to resemble the simple picture of Fig. 2*b* very well. In particular, as indicated by the upper four arrows in Fig. 3, the antibonding states just above the *d* bands are clearly seen. Although these antibonding states are empty on Ni and Pt, they are essentially filled on Cu and Au. On Ni and Pt, the coupling to the metal *d* bands therefore gives rise to an extra attraction on top of the contribution attributable to the interaction with the metal *s* band, but gives rise to a repulsion for the noble metals Cu and Au (ref. 13). Still, however, the bond between the hydrogen atom and the noble metals is not weak—the coupling to the metal *s* band gives rise to a sizeable attraction which is only partly compensated for by the repulsive interaction with the *d* bands—but relative to the bonding in the H_2 molecule, the bond is only marginally stable for Cu and completely unstable for Au.

To see why Au is more noble than Cu, we have to consider the absolute magnitude of the coupling matrix element V_{sd} (and the overlap S_{sd} which is essentially proportional to V_{sd}). The orthogonalization energy between adsorbate and metal *d* states increases monotonically with V_{sd}^2 , which is considerably larger for the 5*d* metals than the 3*d* metals because of the more extended 5*d* states^{17,18}. The orthogonalization energy cost therefore increases down through the groups of the periodic table rendering Au less reactive than Cu.

The concepts emerging from the above H_2 -on-metal calculations now enables a more general discussion of surface nobleness. The important factors are: (1) the degree of filling of the antibonding adsorbate-metal *d* states, and (2) the size of the coupling matrix element. The filling increases to the right in the transition-metal series, and is complete for the noble metals (Cu, Ag and Au); the size of the coupling matrix element increases down through the groups of the periodic table, making the 5*d* metals the most noble. The coupling matrix element also

decreases to the right in the periodic table because the *d* states become more tightly bound as the nuclear charge increases. This is shown in Fig. 4. Although the net result of the two factors does not make Pt very noble in terms of its surface reactivity (hence its use as a catalyst), the net result for Au—which has both a filled antibonding adsorbate-metal *d* state and the largest coupling matrix element—is to make it the most noble metal.

The physical picture presented here for the chemisorbed state also holds for the energy barrier for dissociation. For H_2 at the transition state, the filled molecular bonding σ_g state behaves much the same as the filled H 1*s* state considered above^{9,13}. The molecular antibonding σ_u^* state, however, also interacts covalently with the metal *d* states, and the strength of this interaction completely dominates the trends in the H_2 dissociation barriers over a broad range of transition-metal, noble-metal and alloy surfaces^{15,19}.

The physical picture for the hydrogen/metal-surface interaction is not restricted to hydrogen. Any atom or molecule with a filled one-electron level below the *d* bands will give rise to a repulsive coupling to the *d* states for the noble metals as long as the coupling matrix element is not strong enough as to push the antibonding peak above the Fermi level (compare Fig. 2). Such low-lying states (after the interaction with the metal *s* band has been taken into account) are typical of all the simple gas atoms such as H, C, N and O (ref. 16).

We now turn to the more general nobleness—the ability of the metal to form stable compounds (such as hydrides, oxides and carbides) or to be dissolved. Again the ability to form stable bonds with H, C, N, O and so on must be governed by the processes discussed above. The noble metals, and gold in particular will form the weakest bonds. When a new compound is formed the metal-metal bonds are broken, and this introduces a third factor determining the 'bulk-nobleness', namely the cohesive energy of the metal. This is also included in Fig. 4. It is seen how the 5*d* metals have the largest cohesive energies and they therefore appear more noble in this regard. This in particular explains why Pt is usually considered more noble than Cu despite the fact that Cu surfaces are less reactive than Pt surfaces^{1,5,7}.

In the picture developed above, the nobleness of gold is related to the size of the overlap between the interacting atoms or molecule and the gold *d* states. Clearly, the longer the bond, the smaller the overlap and the less noble gold will appear. This

FIG. 3 The density of one-electron states (DOS) (solid lines) for H atomically chemisorbed on the (111) surface of Ni, Cu, Pt and Au. The DOS is projected onto the atomic H 1*s* state. The surface *d* bands DOS (dashed lines) of the four clean metal surfaces are shown for comparison. The dominant features are the H 1*s*-metal *d* bonding resonances at energies, ϵ , between -5 and -10 eV. Also prominent are the H 1*s*-metal *d* antibonding DOS peaks (indicated by arrows) directly above the metal *d* bands. These antibonding states cause repulsion on Cu and Au, where they are filled. As indicated by the grey-shading, only states below the Fermi energy (which is the energy zero in all cases) are filled.

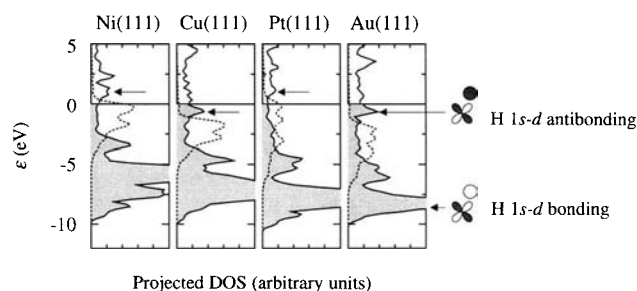
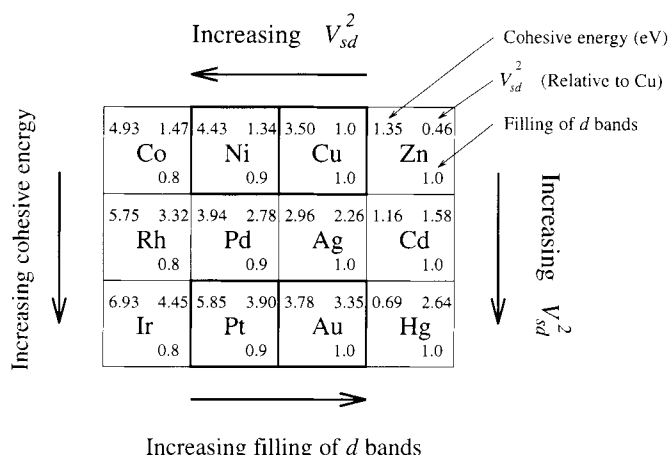


FIG. 4 The s - d coupling matrix element (V_{sd}^2 ; refs 17, 18), the filling of the metal d bands and the cohesive energy²² for metals in the vicinity of gold in the periodic table. The filling of the metal d bands is taken as a measure of the filling of the adsorbate-metal d antibonding state. The largest adsorbate-metal d repulsion, and hence the largest nobleness in terms of the surface reactivity, is obtained by maximizing V_{sd}^2 and having an antibonding state filling of one. This is obtained for gold. The nobleness in terms of ability to resist corrosion and dissolution further involves the cohesive energy of the metals. This energy is largest for the $5d$ metals like Ir and Pt and adds particularly to the nobleness of these metals.



explains why gold is able to form metallic alloys, because the bond lengths here are very long. □

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Latitudinal gradient of atmospheric CO₂ due to seasonal exchange with land biota

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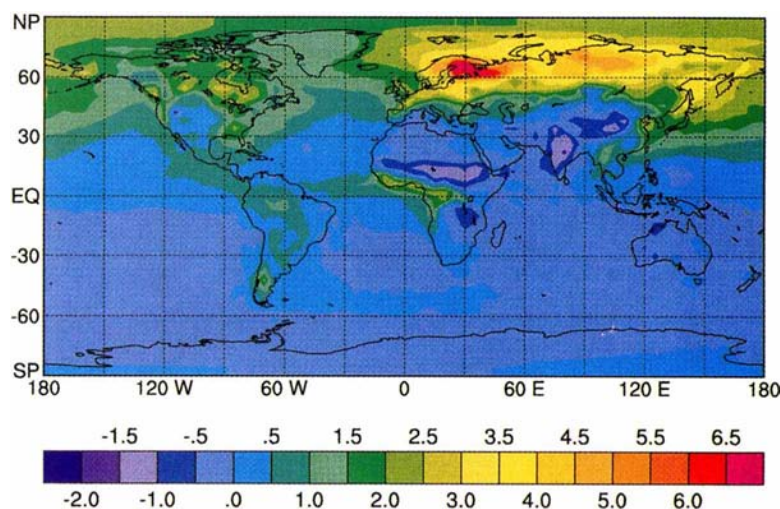
THE concentration of carbon dioxide in the atmosphere is increasing, largely because of fossil-fuel combustion, but the rate of increase is only about half of the total emission rate¹. The balance of the carbon must be taken up in the oceans and the terrestrial biosphere, but the relative importance of each of these sinks—as well as their geographical distribution and the uptake mechanisms involved—are still a matter of debate^{1–4}. Measurements of CO₂ concentrations at remote marine sites^{5–9} have been used with numerical models of atmospheric transport to deduce the location, nature and magnitude of these carbon sinks^{2,10–19}. One of the most important constraints on such estimates is the observed inter-hemispheric gradient in atmospheric CO₂ concentration. Published models that simulate the transport of trace gases suggest that the gradient is primarily due to interhemispheric differences in fossil-fuel emissions, with small contributions arising from natural exchange of CO₂ with the various carbon reservoirs. Here we use

a full atmospheric general circulation model with a more realistic representation of turbulent mixing near the ground to investigate CO₂ transport. We find that the latitudinal (meridional) gradient imposed by the seasonal terrestrial biota is nearly half as strong as that imposed by fossil-fuel emissions. Such a contribution implies that the sinks of atmospheric CO₂ in the Northern Hemisphere must be stronger than previously suggested.

Many previous studies of CO₂ transport in the atmosphere used the transport model developed at the Goddard Institute for Space Studies (GISS) of the National Aeronautics and Space Administration^{2,11–14,17,18}, which prescribes the three-dimensional wind field at four-hour time intervals from the output of an atmospheric general circulation model (GCM). Sub-grid-scale vertical transport of trace gases is simulated in terms of prescribed monthly mean convective frequencies in the parent GCM. Interhemispheric mixing in the model is tuned using an adjustable sub-grid diffusion parameter to produce realistic meridional gradients for tracers with known aseasonal, mid-latitude sources. The annual mean meridional gradient in atmospheric CO₂ simulated by the GISS model is much stronger than observed when the effects of fossil-fuel emissions, seasonal exchange with the terrestrial biota, and emissions from tropical deforestation and from the equatorial oceans are included (about 5 or 6 parts per million by volume (p.p.m.v.) from the Arctic to the Antarctic, as compared to an observed gradient of about 3 or 4 p.p.m.v.)². This implies the existence of sinks of CO₂ in the Northern Hemisphere so strong as to be inconsistent with available oceanographic data². Thus at least some of the Northern Hemisphere sink is believed to be terrestrial, and is thought to be due to direct CO₂ fertilization²⁰, nutrient deposition²¹, reforestation²² and/or climate fluctuations²³.

Although it has long been recognized that atmospheric dispersion of pollutants released at the ground surface depends

FIG. 1 Annual mean mixing ratio of CO_2 (colour scale; p.p.m.v.) in the planetary boundary layer (PBL) as simulated by the Colorado State University (CSU) GCM using surface fluxes representing the purely seasonal exchange with the terrestrial biosphere as calculated in ref. 14 and used in ref. 2. The value at the South Pole has been subtracted at every grid point. The concentration was initialized to be globally uniform, and the model was run on a coarse $7.2^\circ \times 9^\circ$ grid with nine levels for a simulated ten years. The end of this "spin up" run was then used as the initial condition for a further four-year integration on a $4^\circ \times 5^\circ$ grid with 17 levels. All results presented here are for the final three years of the $4^\circ \times 5^\circ$ run. (NP, North Pole; EQ, Equator; SP, South Pole.)



strongly on the depth and intensity of near-surface atmospheric turbulence^{24,25}, this phenomenon has not been resolved in previous simulations of global CO_2 concentration. Because both atmospheric turbulence²³ and the surface flux of CO_2 (ref. 14) are strongly seasonal, correlations between these processes are likely to play an important role in determining the annual mean distribution of atmospheric CO_2 . The GISS tracer model cannot represent such correlations adequately because only monthly mean statistics of convective mixing are employed, and the minimum mixing depth is that of the time-invariant lowest model layer.

We have repeated some of the calculations reported in ref. 2 using the Colorado State University (CSU) GCM²⁶⁻²⁸, in which some important transport processes are represented more realistically than they can be in a tracer model. Surface fluxes of CO_2 were prescribed exactly as in ref. 2, but the tracer calculation was performed on-line in the GCM, at the model time step of six minutes. Penetrative vertical tracer transport by cumulus convection is calculated once per simulated hour. The lowest layer of the model atmosphere is represented as a well mixed planetary boundary layer (PBL), the depth of which is calculated prognostically in terms of the rates of turbulent entrainment and turbulence kinetic-energy dissipation, and loss of mass into cumulus clouds²⁹. Emissions of CO_2 from the surface are assumed to be immediately vertically mixed in this layer, and exert a greater effect on the PBL concentration under stable conditions when the PBL is shallow than under unstable conditions when the PBL is deep. A comparison of the seasonal variations of the simulated depth of the PBL and other near-surface meteorological variables in the GCM with the limited observational data available shows good agreement³⁰. The interhemispheric gradient of ^{85}Kr and vertical profiles of ^{222}Rn simulated by the CSU GCM agree quite well with observations³¹ without the use of any tunable diffusion such as that used in the GISS model.

The seasonal drawdown of atmospheric CO_2 by photosynthesis and subsequent release of CO_2 by microbial respiration involve fluxes that are stronger than the anthropogenic emission rate by about an order of magnitude¹. These fluxes account for the very large seasonal amplitude of the annual cycle of CO_2 concentrations in the Northern Hemisphere^{1,10-19}. Although the prescribed seasonal CO_2 fluxes at the land surface are exactly balanced over an annual cycle¹⁴ (the net annual flux is zero at every grid point), they result in a non-uniform annual mean concentration field at the surface (Fig. 1). This effect has been noted by others, and has been attributed to the covariance of seasonal CO_2 exchange with meridional transport^{11,12} and with convective mixing^{12,18}. In the CSU GCM, the effect is enhanced

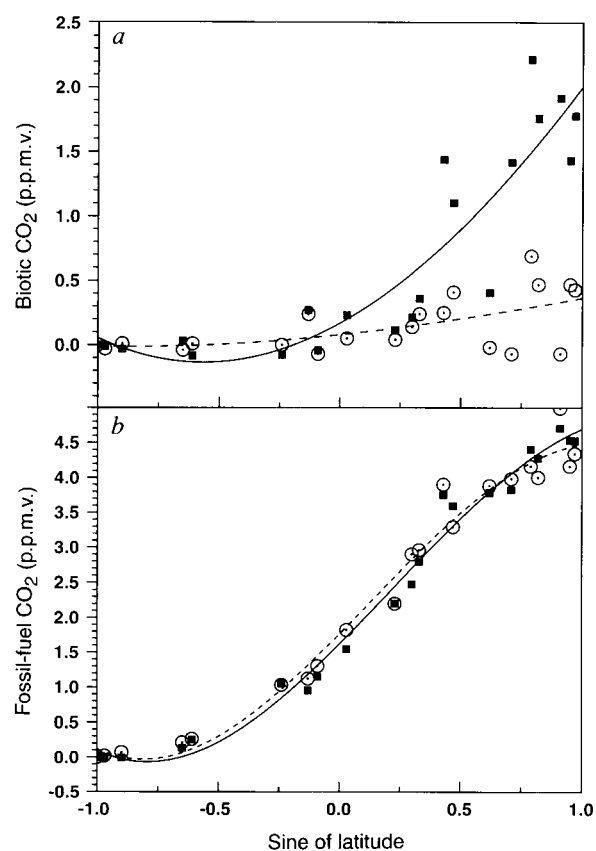


FIG. 2 Annual mean mixing ratio (p.p.m.v.) of simulated CO_2 ; a, using the seasonal biotic fluxes of ref. 2, and b, using the fossil-fuel emission estimates of ref. 2. Values are annual means in the PBL at the grid cells containing the 21 flask-sampling stations used in ref. 2. Where the grid cell was defined as land, we chose the adjacent cell offshore because flask-sampling protocol selects marine air only. Filled squares indicate values simulated by the CSU GCM, and open circles with a central dot indicate values simulated by the Goddard Institute for Space Studies (GISS) tracer model. The curves are cubic polynomials fitted by least-squares to the station values (the solid curves were fitted to the CSU GCM results, and the dashed curves were fitted to the GISS results). The value at the South Pole has been subtracted.

by the correlation between the seasonal CO_2 flux and vertical mixing by PBL turbulence. In regions with strongly seasonal vegetation, the CO_2 flux (to the atmosphere) is strongly negative at the peak of the summer growing season when both PBL turbulence and cumulus convection are active and vigorous, so air that has been depleted of CO_2 tends to be carried aloft. By contrast, in spring and autumn when CO_2 emissions from soil respiration exceed photosynthetic uptake, vertical mixing is weak or inactive, so the CO_2 -enriched air is confined to a thin layer close to the ground. This seasonal cycle gives rise to a net positive concentration anomaly in the annual mean.

At the remote marine locations sampled by the flask network⁹, the Arctic-to-Antarctic CO_2 gradient imposed by the purely seasonal fluxes at the land surface in the CSU GCM is more than four times as strong as the gradient simulated by the GISS tracer model (Fig. 2a). The difference in meridional gradients between the two models does not result from weaker meridional transport, as the simulated concentrations of fossil-fuel CO_2 at the locations of the flask stations are nearly identical (Fig. 2b). The simulated concentration fields arising from the fossil-fuel emissions and the seasonal vegetation are the subject of a tracer transport model intercomparison project (TRANSCOM), which is nearing completion (P. Rayner, personal communication). The meridional gradients simulated by the other models in the study vary considerably; the results presented here are within the range found by other participants in this project (unpublished results).

The difference between the simulated biogenic CO_2 concentrations in the two models is largest in the middle to high latitudes of the Northern Hemisphere (Fig. 2), where biotic CO_2 exchange with the atmosphere is most strongly seasonal¹⁴. Seasonal variations in biotic CO_2 flux and simulated vertical tracer transport by both cumulus convection and PBL turbulence are negatively correlated over this region (Fig. 3), and at the locations with the highest annual mean CO_2 concentrations, both of these cor-

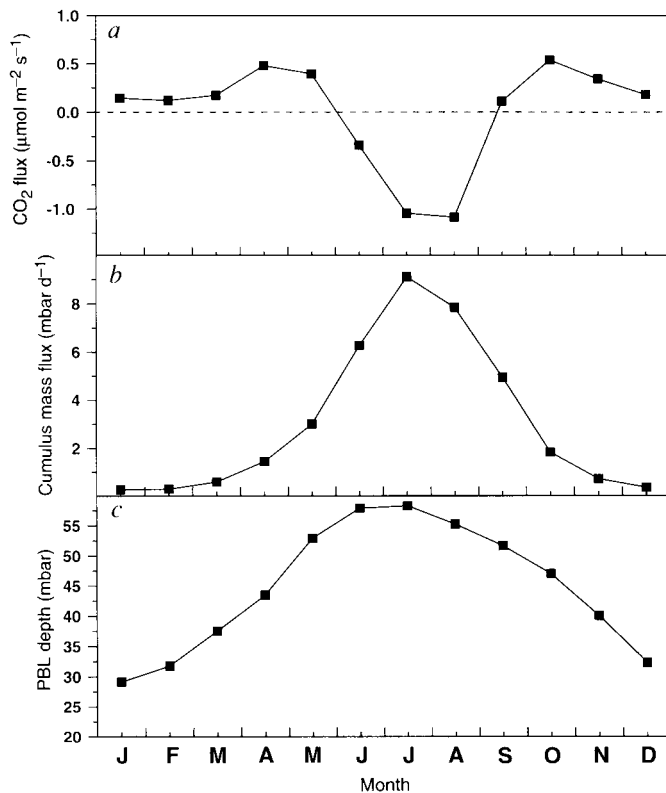


FIG. 3 Seasonal variations; a, of prescribed surface flux of CO_2 from the terrestrial biosphere to the atmosphere, b, of simulated atmospheric mass flux due to cumulus convection at the top of the atmospheric PBL, and c, of simulated depth of the atmospheric PBL. Values are area-weighted means for land points north of 28°N , averaged for each calendar month.

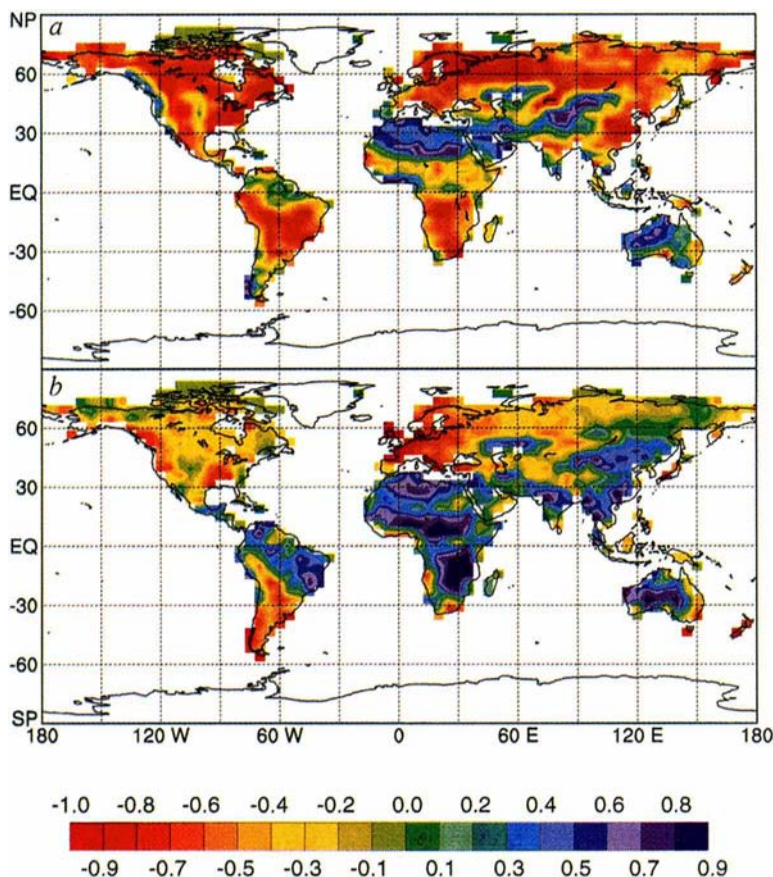


FIG. 4 Correlation coefficients (colour scale) of the relationship between prescribed monthly mean terrestrial surface CO_2 flux and; a, simulated cumulus mass flux at the top of the PBL, b, simulated depth of the PBL. The correlation was calculated separately at each land grid cell in the model, and the resulting values were contoured to produce the maps shown. Note that the colour scheme has been reversed (red indicates negative values and blue indicates positive values) for easier comparison with Fig. 1.

relations are very strong (Fig. 4). Over the tropics, where only cumulus convection is negatively correlated with CO₂ flux, the concentration is much lower. Because Fig. 4 presents the correlation rather than the covariance between the variables, the values vary independently of the magnitude of the CO₂ flux, and are therefore high in some regions (such as the Sahara) with little biological activity. These regions are characterized by minimal CO₂ concentration anomalies in the annual mean (Fig. 1). In view of the fact that PBL turbulence is not represented adequately in the GISS model, we conclude that the variable-depth PBL of the GCM has introduced a greater degree of correlation between the surface fluxes and vertical mixing over the temperate and boreal continents, and that this greater correlation has resulted in the higher annual mean CO₂ concentrations simulated for those regions.

Using the CSU GCM, the simulated meridional gradient in CO₂ concentration imposed by seasonal exchanges with the terrestrial biota (~2 p.p.m.v from pole to pole) is nearly half as strong as that imposed by the fossil-fuel emissions (~4.5 p.p.m.v.). This implies that the sinks of atmospheric CO₂ in the Northern Hemisphere must be even stronger than previously estimated². Calculation of the global carbon budget would require simulation of the effects of air-sea exchange, land use, and other sources and sinks, and is beyond the scope of this work, but the effect of the enhanced meridional gradient arising from the seasonal biotic fluxes would be significant. To illustrate the sensitivity of the calculated budget to the enhanced biogenic gradient, we substituted the CSU GCM results for the GISS results in the calculations of ref. 2. This leads to an 'extra' 1.5 p.p.m.v of CO₂ at high northern latitudes that is not observed and must be offset without violating the observed annual mass balance of the atmosphere. The excess CO₂ could be offset by an additional Northern Hemisphere sink of 1.2–2.5 Gt C yr⁻¹ (depending on the proximity of the sink to the flask stations) relative to ref. 2. Such a sink would have to be compensated by an equatorial source of the same magnitude, yielding a deforestation rate of 2–3.5 Gt C yr⁻¹, which exceeds current estimates for this source¹. Alternatively, the stronger meridional gradient in CO₂ concentration simulated by the CSU GCM could be compensated by an extra sink of 0.6 Gt C yr⁻¹ in the Northern Hemisphere, and a Southern Hemisphere sink that is weaker by the same amount. This would yield a sink strength of only 1.0–1.2 Gt C yr⁻¹ for the ocean gyres in the Southern Hemisphere². □

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Atmospheric inputs of dissolved organic nitrogen to the oceans

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THE input of fixed nitrogen to the oceans by *in situ* fixation, river/groundwater supply and atmospheric deposition represents an important control on marine productivity on long timescales, and hence on ocean-atmosphere CO₂ exchange and its effects on climate^{1–3}. Any assessment of human perturbation of the global nitrogen cycle also requires an accurate estimate of these inputs. The current best estimates suggest that the natural fluvial and atmospheric inputs are of similar magnitude^{3,4}, and that globally both have been increased by a factor of two above natural levels as a result of human activity^{3–5}. Dissolved organic nitrogen represents more than half of the fluvial input of dissolved fixed nitrogen, but current estimates of atmospheric inputs are usually based on only the inorganic (NO₃⁻ + NH₄⁺) component, although some authors have recognized the potential importance of organic nitrogen^{6–9}. Here we present analyses of dissolved organic nitrogen in rain and snow which show that it is a ubiquitous and significant component of precipitation, even in remote marine areas. Our results require an approximate doubling of present estimates of the atmospheric input of fixed nitrogen to the oceans, and an increase in estimates of the total fixed-nitrogen input by a factor of about 1.5. These results indicate that the human impact on the global nitrogen cycle may be larger than has been thought.

Rainwater samples from various locations have been collected and analysed for nitrate, ammonium and dissolved organic nitrogen (DON) (Table 1). The first and clearest conclusion of this study is that, although average concentrations vary considerably between locations, DON is a ubiquitous component of rain water, and in remote marine locations it is the dominant form of fixed nitrogen. Atmospheric fixed-nitrogen budgets must clearly include DON. This conclusion raises the question of the origin—whether marine or terrestrial—and the composition of this DON.

The chemical composition of atmospheric, and marine, organic matter is largely unknown^{10,11}. At continental sites, atmospheric DON seems to be a biologically available nitrogen source with urea an important constituent¹². Other studies report half the reduced nitrogen in aerosols to be organic and relatively

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TABLE 1 DON and DIN concentrations found in this study

Sample	Site type	Sample type	Number of samples	DIN† (μM)	DON‡ (μM)
UEA (UK)	Urb./Ru.	Rain	36	69±11	18±4
Czech Rep.	Ru.	Snow	5	19±5	7±3
N. Carolina	Ru.	Rain	11	34±24	9±2
Maraba (Amazonia)	Ru.*	Rain	6	77±53	22±4
Recife (Brazil)	Ru.†	Rain	11	9±3	3±1
Bermuda	Re.	Rain	18	11±2.5	16±7
Tahiti	Re.	Rain	16	2.5±0.3	13±2
NE Atlantic					
Approx. 20° W	Re.‡	Rain			
50–58° N			9	5±4	8±2.5
32–50° N			4	3±3	6±3

Sampling sites are classified as urban (Urb.; close to large population centres), rural (Ru.; ≤ 500 km from large population centres) and remote (Re.; ≥ 500 km from large population centres). Dissolved inorganic nitrogen (DIN) is the sum of NO_3^- and NH_4^+ concentrations; the concentrations of dissolved organic nitrogen (DON) and DIN are given as 'mean $\pm$ s.d.'

Rain samples were collected either as 'wet-only' samples using automatic rain collectors, or using manual collectors opened and closed at the beginning and end of rain events or left open overnight. The maximum time for dry deposition was thus ~ 12 h and we consider our estimates to be essentially of wet deposition only. The Czech snow samples were collected from a snow pit and were thus subject to dry deposition. In general samples were frozen for storage, although at some sites this was not possible (Tahiti and Bermuda) and HgCl_2 was used as a preservative. We have no evidence of large-scale nitrogen losses during storage by either procedure. The precision of results for the analysis of inorganic and total nitrogen on replicate fresh samples (within 24 h of collection) and on samples analysed over periods of up to a year are similar ($\pm 15\%$ or better) with no evidence of systematic trends. Samples have been filtered immediately on arrival at Norwich using pre-ashed Whatman GF/F filters, so results represent DON nominally < 0.7 μm. Samples were analysed by the ultraviolet photo-oxidation method³⁰ which may underestimate DON; hence our results represent lower limits¹⁰. However, our results for Bermuda rains are on average higher than previously reported⁷ using a persulphate oxidation technique (Tables 1 and 2) and recoveries of organic nitrogen standards, urea (as recommended by ref. 10), glycine, imino-diacetic acid and methionine, are 98–100%. Thus we believe any underestimation of DON is modest and does not affect our conclusions. At remote sites where NO_3^- and NH_4^+ concentrations are low, precision of our DON estimates is better than $\pm 15\%$ and full procedural blanks (including sampling) were < 0.6 μM of total nitrogen. Because DON is determined by difference, precision is worse in samples with high inorganic nitrogen and low DON³⁰.

* This site is in an area of year-round biomass burning.

† This site is coastal and subject to onshore winds most of the time. Because of its proximity to a major land mass we have chosen not to include it with remote marine sites.

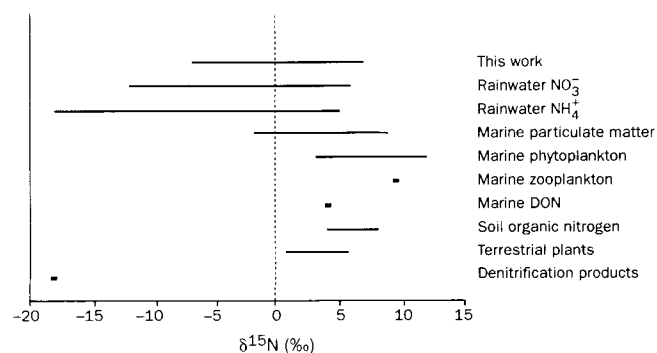
‡ Shipboard sample collections on two cruises, samples from 50–58° N, stored frozen and unfiltered but filtered before analysis.

involatile^{13,14}. In marine rains, high concentrations of dissolved free amino acid and total protein have been reported¹⁵, although other workers found rather lower concentrations¹⁶ possibly because of differences in sampling procedures¹⁷. Three of our rain samples (University of East Anglia (UEA), Tahiti and North Atlantic) were analysed for methylamines; these compounds were found to comprise $< 1\%$ of the total DON¹⁸. We have used ultrafiltration on ten of our largest samples (three UEA, two Tahiti and five Bermuda) to estimate the relative molecular mass (M_r) of the DON. We found that $< 10\%$ of the DON has $M_r > 5,000$, consistent with results for dissolved organic carbon in North American rain water¹⁹. We suggest the DON reported here is therefore relatively low-molecular-weight material which will include dissolved amines and hence is likely to be bioavailable, at least in part.

The gradient of DON from rural to remote regions is much less marked than for dissolved inorganic nitrogen (DIN; Table

1) whose constituent species are thought to be derived, even in remote areas, mainly from anthropogenic sources, such as nitrate from combustion processes in urban areas²⁰. Indeed DON shows no strong correlation with other rainwater components of either terrestrial (for example, NO_3^- ; $r = 0.15$, $n = 118$) or marine origin (for example, Na^+ ; $r = -0.04$, $n = 74$, excluding data below detection). However, high DON concentrations do seem to be most common in continental rains. Thus 45% of UEA samples and 60% of central Amazonia (Maraba) samples have DON concentrations of > 20 μM, compared to 8% from the sites exposed to marine air (Recife, Bermuda, Tahiti and northeast Atlantic). This observation, together with the high DON concentrations found in Ontario rain (Table 2) suggests a continental source for DON. The low concentrations of DON at the Czech site (Table 1) may reflect losses during partial melting of lying snow^{21,22} or reduced biogenic sources during winter, consistent with the Ontario data (Table 2).

FIG. 1 $\delta^{15}\text{N}$ values for DON in rain water from this study and representative values for potential DON sources from other recent studies; rainwater nitrate^{24,26,34,35}; rainwater ammonium^{24,26,34}; marine suspended particulate matter^{36,37}; marine phytoplankton³⁸; marine zooplankton³⁸; marine DON—model estimate³⁷; soil organic nitrogen²⁶; terrestrial plants³⁸; gaseous products of denitrification (NO and N_2O)²⁴. Note $\delta^{15}\text{N}$ of NO_x from vehicle emissions is -13 to -2% ; from coal combustion $+6$ to $+13\%$ (ref. 25); $\delta^{15}\text{N}$ of anthropogenic NH_3 is -16 to $+1\%$ (ref. 26). $\delta^{15}\text{N}$ of DON in rain water was measured as follows ($\delta^{15}\text{N} = [(^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{atmosphere}} - 1]$ per mil). Nitrate and ammonium were quantitatively removed on ion-exchange resins³⁹ before oxidation by ultraviolet light. The resulting nitrate and ammonium was reduced to ammonia with Devarda's alloy, distilled into dilute sulphuric acid and evaporated. The resulting ammonium sulphate was combusted in sealed tubes with Cu/CuO ; the nitrogen gas formed was purified and analysed with a Sira Series 2 isotope mass spectrometer. Based on the analysis of urea and mixed amino acids with and without this treatment, the method is accurate and precise to better than $\pm 0.2\%$.



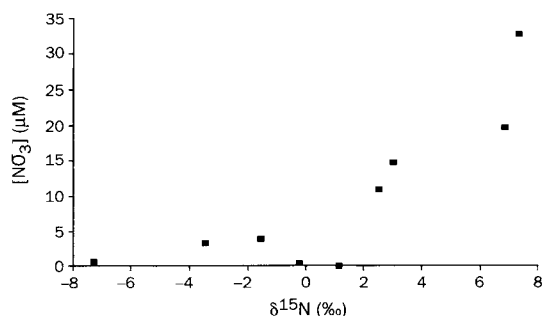


FIG. 2 Plot of $\delta^{15}\text{N}$ of DON in rain versus nitrate concentration for samples from UEA ($n=3$), Tahiti ($n=3$), Maraba ($n=1$) and Recife ($n=2$). Lower nitrate concentrations indicate rains from remote sites subject to the least anthropogenic perturbation. A plot of $\delta^{15}\text{N}$ against ammonium concentration yields a similar relationship because ammonium and nitrate are highly correlated ($r=0.93$ for these samples).

A potential seawater source of DON is bubble bursting and enrichment of the resultant aerosol with seawater DON. The average Na^+ of all our rains is $172\ \mu\text{M}$ which, together with an average DON in sea water of $7\ \mu\text{M}$ (ref. 10) would require an enrichment factor for DON in marine aerosols over sea water of about 5×10^3 to explain the observed DON in rain water. This is a factor of 2–20 times the enrichments observed for organic carbon²³ in seasalt aerosol, and hence we consider a dominant seawater source to be unlikely.

To try to elucidate sources further, we have measured nitrogen isotope abundances ($\delta^{15}\text{N}$; see Fig. 1 legend) of DON on the larger-volume rain samples. These $\delta^{15}\text{N}$ values range from -7.27 to $+7.32\text{‰}$ (Fig. 1) with a trend for the lowest $\delta^{15}\text{N}$ to be in rain from the more remote sites (Fig. 2). Soil organic matter, continental plants and marine nitrogen sources all tend to be isotopically heavier (Fig. 1), and hence unlikely to be the exclusive source of rainwater DON at these remote sites, unless there is extensive isotopic fractionation during organic nitrogen formation and transport. The $\delta^{15}\text{N}$ of rainwater nitrate and ammonium covers the range seen for DON (Fig. 1), whereas the gaseous products of denitrification have very low $\delta^{15}\text{N}$ values²⁴. To achieve the observed DON $\delta^{15}\text{N}$ at the remote sites seems to require a significant contribution from anthropogenic NO_x and NH_3 (refs 25, 26) or from the products of denitrification. At the continental sites, the heavier isotopic signature is consistent with an additional soil or plant source for the DON.

This evidence cannot conclusively identify the source of DON, but the higher concentrations in continental rains, the need for a relatively large sea-surface fractionation to sustain a marine source and the isotope results all suggest that a continental source is more likely. The low spatial gradients are similar to those for aerosol organic carbon which are ascribed to slow gas-to-particle conversions of low-molecular-weight organic compounds from continental sources^{23,27}. We suggest DON is similarly formed or produced by low-temperature reactions of NH_3 and NO_x with soot or other organic matter probably close to sources²⁸. During these reactions and subsequent oxidation, the aerosol organic component could also become relatively water soluble and hence more effective cloud condensation nuclei—a process which has been argued to be of global significance in climate regulation¹¹.

The argument for a terrestrial DON source is strengthened by recent reports of organic nitrogen emissions during biomass burning, in addition to emissions of NO_x , NH_3 , HCN , CH_3CN and N_2 (ref. 29). Industrial combustion sources could also release DON via reactions of soot with NO_x and NH_3 (ref. 28). Such diverse combustion sources would contribute to the widespread distribution of DON.

TABLE 2 DON concentration found in other studies

Site	DON (μM)	Reference
Bermuda	7.5	8
North Sea	6.3	30
New Zealand	9	12
Japan	17	12
Venezuela	13	31
Ontario	8 (winter snow)	21
	37 (summer rain)	21
Florida	29	32

Mean concentrations of DON in rain water reported in other studies from rural and remote areas.

Our data suggest a global background rainwater DON concentration of 6–16 μM at remote marine sites (Bermuda, Tahiti and North Atlantic). Using a global annual precipitation rate⁴ to the oceans of $360 \times 10^{12}\ \text{m}^3$, we estimate a global wet depositional DON flux of $(2\text{--}6) \times 10^{12}\ \text{mol yr}^{-1}$ compared to an atmospheric DIN flux of $(2\text{--}5) \times 10^{12}\ \text{mol yr}^{-1}$ (refs 2–4). Thus the inclusion of DON approximately doubles estimates of atmospheric fixed-nitrogen fluxes to the oceans.

To assess the effect of DON on the marine nitrogen budget, we present in Table 3 best estimates of fixed-nitrogen inputs to the oceans. All fluxes have large uncertainties associated with them, but it is clear that DON is an important source of fixed nitrogen to the oceans. We estimate that the inclusion of atmospheric DON increases estimates of fixed-nitrogen inputs to the oceans from $(4.5\text{--}13) \times 10^{12}\ \text{mol yr}^{-1}$ to $(6.5\text{--}19) \times 10^{12}\ \text{mol yr}^{-1}$ with the atmosphere probably being the dominant source (Table 1). Further work is clearly needed to elucidate the origin and bioavailability of this DON.

If rainwater DON is of natural origin, it implies that the nitrogen input to the pre-industrial ocean is substantially larger than previously estimated and must be balanced by either higher long-term denitrification or carbon burial rates.

If, as our data seem to imply, the DON is of anthropogenic origin, we estimate that human effects have increased fixed-nitrogen fluxes to the ocean from $(3\text{--}8) \times 10^{12}\ \text{mol yr}^{-1}$ to $(6.5\text{--}19) \times 10^{12}\ \text{mol yr}^{-1}$, implying an even more massive perturbation of the global nitrogen system than previously believed. In some previous considerations of the effect of human perturbations of the global nitrogen cycle, the effect on the coastal zone has been emphasized². The results reported here indicate the importance of atmospheric transport, the effect of which is not focused on the coastal zone. To date, estimates of the effects of atmospheric inputs on the oceans have concentrated primarily on DIN and it is clear that DON should be included in such estimates^{6,7}. The potential effects of this large increase in fixed-nitrogen inputs

TABLE 3 Estimates of present-day rates of fixed-nitrogen inputs to the oceans

Source			Nitrogen flux ($10^{12}\ \text{mol yr}^{-1}$)
Dinitrogen fixation ^{2,33}			1–3†
River input	DIN+DON ^{3,4}	Natural	1–2.5
		Anthropogenic	0.5–2.5
Atmospheric input	DIN ^{3,4}	Natural	1–2.5*
		Anthropogenic	1–2.5
	DON	(this study)	2–6

Ranges given in some cases reflect the real ranges of values observed (atmospheric DON, river input) but in other cases refer to the different single-value best estimates by the relevant authors.

* Nitrate in rain water is estimated to be 50% anthropogenic on a global scale⁴ and we assume ammonia is similarly 50% anthropogenic as in ref. 3.

† Higher estimates (to $14 \times 10^{12}\ \text{mol yr}^{-1}$) have been reported but are considered speculative³.

from human activities include increased marine primary productivity and, if sustained in the long term, increased carbon burial. □

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Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria

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GLUCOSE-6-PHOSPHATE dehydrogenase (G6PD) deficiency, the most common enzymopathy of humans, affects over 400 million people¹. The geographical correlation of its distribution with the historical endemicity of malaria suggests that this disorder has risen in frequency through natural selection by malaria^{2,3}. However, attempts to confirm that G6PD deficiency is protective in case-control studies of malaria have yielded conflicting results^{4–8}. Hence, for this X-linked disorder, it is unclear whether both male hemizygotes and female heterozygotes are protected or, as frequently suggested, only females^{1,5,11}. Furthermore, how much protection may be afforded is unknown. Here we report that, in two large case-control studies of over 2,000 African children, the common African form of G6PD deficiency (G6PD A–) is associated with a 46–58% reduction in risk of severe malaria for both female heterozygotes and male hemizygotes. A mathematical model incorporating the measured selective advantage against

malaria suggests that a counterbalancing selective disadvantage, associated with this enzyme deficiency, has retarded its rise in frequency in malaria-endemic regions. Although G6PD deficiency is now regarded as a generally benign disorder, in earlier environmental conditions it could have been significantly disadvantageous.

Despite the extensive heterogeneity of G6PD deficiency, a single molecular variant, G6PD A–, seems predominant in sub-Saharan Africa^{12–14}. It differs from the normal G6PD B allele at two amino-acid positions, one at position 376 encoding the B to A change and the other at position 202 that differentiates the A allele with 85% enzymatic activity from the A– allele with 12% activity^{12–14}. Previous clinical studies of G6PD and malaria attempted to define the various genotypes from phenotypic measurements of enzyme levels, electrophoretic mobility and cytochemical staining patterns^{4–6}. This is difficult because overlapping levels of enzyme activity are seen among genotypes, partly related to variable inactivation of X chromosomes in female heterozygotes, and partly to altered rates of erythrocyte turnover in acute malaria¹⁵. The largest study suggested that female heterozygotes, but not male hemizygotes, may be protected based on parasite density measurements in Nigerian children with acute malaria⁵. Although an ingenious mechanism has been proposed to explain how only female heterozygotes might be protected¹⁶, data supporting this mechanism have not been confirmed^{17,18} and the issue of a sex difference in protection has remained unresolved^{5–11}. Recently, it has been found that comparison of genotype frequencies in children with the relatively rare condition of complicated or severe malaria^{19,20} with those of matched controls seems to offer a more sensitive measure of the effect of malaria resistance alleles^{21–23}. Earlier studies had compared parasite densities between genotypes in children with the more common clinical condition, uncomplicated or mild malaria. Hence, here we use DNA analysis to define genotypes and, in our primary analysis, we have studied G6PD genotype frequencies in children with life-threatening severe malaria in comparison with local non-malaria controls.

We have determined the frequencies of the G6PD A– allele in two large case-control studies of *Plasmodium falciparum* malaria in West and East Africa, and Table 1 shows the frequencies of individuals with different genotypes in the various clinical groups. The frequency of female heterozygotes was lower in the children with severe malaria than in controls in both studies.

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TABLE 1 Frequencies (percentages) of female G6PD A- heterozygotes and male hemizygotes in The Gambia and Kenya

	Controls	Mild malaria	Severe malarial anaemia	Cerebral malaria	All severe malaria	Odds ratio (confidence interval), mild v. controls	Odds ratio (confidence interval), severe v. controls
Females heterozygotes							
Gambia	13.7 <i>n</i> = 182	9.1 <i>n</i> = 165	5.3 <i>n</i> = 94	6.8 <i>n</i> = 174	6.7 <i>n</i> = 255	0.62 (0.30–1.29) <i>P</i> = 0.23	0.45 (0.22–0.89) <i>P</i> = 0.02
Kenya	27.3 <i>n</i> = 143	17.2 <i>n</i> = 116	20.6 <i>n</i> = 68	16.9 <i>n</i> = 65	18.8 <i>n</i> = 133	0.52 (0.27–0.99) <i>P</i> = 0.047	0.60 (0.33–1.11) <i>P</i> = 0.11
Combined odds ratio						0.59 (0.36–0.94) <i>P</i> = 0.027	0.54 (0.34–0.84) <i>P</i> = 0.006
Male hemizygotes							
Gambia	5.9 <i>n</i> = 239	2.7 <i>n</i> = 182	1.3 <i>n</i> = 80	1.9 <i>n</i> = 208	1.4 <i>n</i> = 279	0.45 (0.14–1.38) <i>P</i> = 0.20	0.23 (0.06–0.77) <i>P</i> = 0.012
Kenya	18.8 <i>n</i> = 149	14.3 <i>n</i> = 133	14.9 <i>n</i> = 47	8.6 <i>n</i> = 70	11.1 <i>n</i> = 117	0.72 (0.23–1.42) <i>P</i> = 0.40	0.54 (0.25–1.15) <i>P</i> = 0.12
Combined odds ratio						0.63 (0.36–1.11) <i>P</i> = 0.12	0.42 (0.22–0.77) <i>P</i> = 0.004

The primary comparison was of severe malaria cases with non-malaria controls. The odds ratios for this comparison and for the comparison of mild malaria with controls are shown with 95% confidence intervals (in parentheses) and two-tailed significance (*P*) values. There was no significant heterogeneity in odds ratios between the two populations studied, so combined odds ratios and significance levels were calculated. These indicate that female heterozygotes and male hemizygotes were both significantly less frequent in the severe malaria group. Too few homozygous females were identified in either population (1 control in The Gambia; in Kenya 6 controls, 3 mild and 4 severe malaria cases) to allow measurement of the susceptibility or resistance of this genotype to malaria. A possible protective effect of the G6PD A genotype, with 85% of normal enzyme activity, was sought by comparing the frequencies of this genotype between the clinical groups in which an effect was perhaps most likely to be detectable, that is, males with severe malaria, with control males. But this genotype did not differ in frequency between the severe malaria cases (22.2% in The Gambia, 24.4% in Kenya) and the control group (21% and 22.5% respectively) in either country. **Group characteristics.** Clinical evidence of haemolysis during severe malaria is rare in both populations and no difference in haemoglobin levels (g dl⁻¹, mean $\pm$ s.e.m.) was found between G6PD genotypes (for example, for Kenya: in normals, males and females without the A- allele, 7.94 ± 0.11 ; in heterozygotes 8.07 ± 0.32 ; in hemi- or homozygotes 7.78 ± 0.29). Heterozygosity for haemoglobin S was strongly protective against both severe and mild malaria in both areas but no clear evidence of interaction between G6PD and haemoglobin S genotypes was observed, although the power of the study to detect this was low (results not shown). G6PD enzyme levels measured at recruitment, in Kenya only, showed a correlation of enzyme level (in units per 10¹² red blood cells, mean $\pm$ s.e.m.) with G6PD genotype (normals 292 ± 7 ; heterozygotes 238 ± 14 ; hemi- or homozygotes 141 ± 16), but no significant difference in mean enzyme activity was observed between clinical groups (controls 277 ± 9 ; mild malaria 249 ± 10 ; severe malaria 289 ± 12). Parasite densities (geometric mean with 95% confidence interval $\times 10^3 \mu\text{l}^{-1}$) in malaria cases (studied within 2 h of arrival at hospital) in The Gambia did not differ significantly between genotypes (normal 75.8, 67.1–85.6; heterozygotes 116.8, 65.9–207.0; hemizygotes 42.1, 6.6–266.8). In Kenya, the parasite densities in hemizygotes (45.4, 31.8–64.7) was lower than in heterozygotes (98.0, 67.4–142.5; *P* < 0.01) and normals (79.7, 70.3–90.4; *P* < 0.01). **Design.** Children up to 10 years of age were studied in two regions of endemic malaria. The Gambian children were recruited near to the capital Banjul as part of a large case-control study of severe malaria described previously²¹. The Kenyan cases were recruited at Kilifi District Hospital on the Kenyan coast and matched for age and area of residence to health community controls, as part of a larger case-control study described in detail elsewhere (K. Marsh et al., in preparation). Ethical approval was granted, for the Gambian study, by the MRC/Gambian government joint ethical committee and, for the Kenyan study, by the KEMRI ethical committee. The Gambian control children consisted of inpatients and outpatients with a variety of non-malarial illnesses and without *P. falciparum* parasitaemia who were matched for age and location as a group to the severe malaria cases. The Kenyan control group consisted of healthy community children, recruited irrespective of the presence or absence of asymptomatic parasitaemia, who were individually matched for age and location to the cases of severe and mild malaria. Cases of severe malaria were classified as either cerebral malaria or severe malarial anaemia; a small minority of children had both conditions. The criteria for inclusion in these clinical categories were as reported^{21,22}, except that in Kilifi the threshold parasite density deemed appropriate for inclusion in the severe malarial anaemia group was 10,000 μl^{-1} with a haemoglobin level <5 g dl⁻¹ (but 2,500 μl^{-1} in The Gambia), reflecting the higher transmission of malaria in the Kilifi area than in the Gambian study area. The severe malaria cases were slightly older in The Gambia (mean 36 months) than in Kenya (mean 27 months). **Statistical analysis.** Comparisons between clinical groups were made using the χ^2 test with continuity corrections. Odds ratios were calculated and are shown with 95% confidence intervals. The possible confounding effects of ethnic group heterogeneity in The Gambia on the odds ratio was assessed using a Mantel-Haenszel test. χ^2 tests for heterogeneity showed no significant differences between the odds ratios in the Gambian and Kenyan studies. Combined odds ratios were therefore calculated to provide pooled estimates, which are shown with 95% confidence intervals. Comparison of parasite density between groups was performed using t-tests on log-transformed data. **Methods.** Haemoglobin levels and parasite densities were measured by standard methods. Haemoglobin S was detected by cellulose acetate electrophoresis. G6PD enzyme levels were measured spectrophotometrically using Sigma kit 345-B. The higher than expected enzyme activity detected in the enzyme-deficient individuals using this assay is unexplained. Genomic DNA was analysed by polymerase chain reaction (PCR) amplification and oligonucleotide hybridization. Preliminary studies showed that the most prevalent molecular type of the G6PD A- allele appeared to account for the great majority of deficiency alleles in males in each population. We therefore compared frequencies of this variant, containing a guanine to adenine substitution at nucleotide 202, using a PCR amplification of a 109-base fragment (nucleotides 158–267) using primers 5'-GTGGCTGTTCCGGGATGGCCTTCTG-3' and 5'-CTTGAAGAAGGGCTCACTCTGTTTG-3' and 30 cycles of denaturation at 94 °C (45 s), annealing at 65 °C (60 s) and extension at 72 °C (120 s). Alleles were distinguished by hybridization with radiolabelled oligonucleotide probes 5'-TTCATCATGGGCTA-3' (G6PD A-) and 5'-TTCATCGTGGGCTA-3' (G6PD non-A-) with washing at 36 °C and 38 °C respectively. G6PD A alleles were distinguished from G6PD B by using the primers and amplifications conditions described¹⁴ and hybridization with the probes 5'-GCCACATGGATGCCCTC-3' (G6PD A) and 5'-GCCACATGAATGCCCTC-3' (G6PD B), with washing at 54 °C and 52 °C, respectively.

Analysis of the combined data sets was performed to provide an overall estimate of the odds ratio and indicated that this genotype was associated with about 46% protection against severe malaria (odds ratio (OR) 0.54 (95% confidence interval (c.i.) 0.34–0.84), *P* < 0.006) and also with significant protection against mild malaria (OR 0.59 (95% c.i. 0.36–0.94), *P* < 0.03). Male hemizygotes were also less frequent among the children with severe malaria, with the combined odds ratio indicating

that this genotype was associated with about 58% protection (OR 0.42 (95% c.i. 0.22–0.77), *P* < 0.005). The decrease in frequency of this genotype among the cases of mild malaria was not statistically significant (OR 0.63 (c.i. 0.36–1.11), *P* = 0.12). Female G6PD A- homozygotes were too rare to allow measurement of the susceptibility of this genotype to severe malaria. We also determined the frequencies of the normal G6PD B allele and the very mildly deficient G6PD A allele in the clinical groups

and found no difference in risk between male genotypes containing B and A alleles (see Table 1 legend).

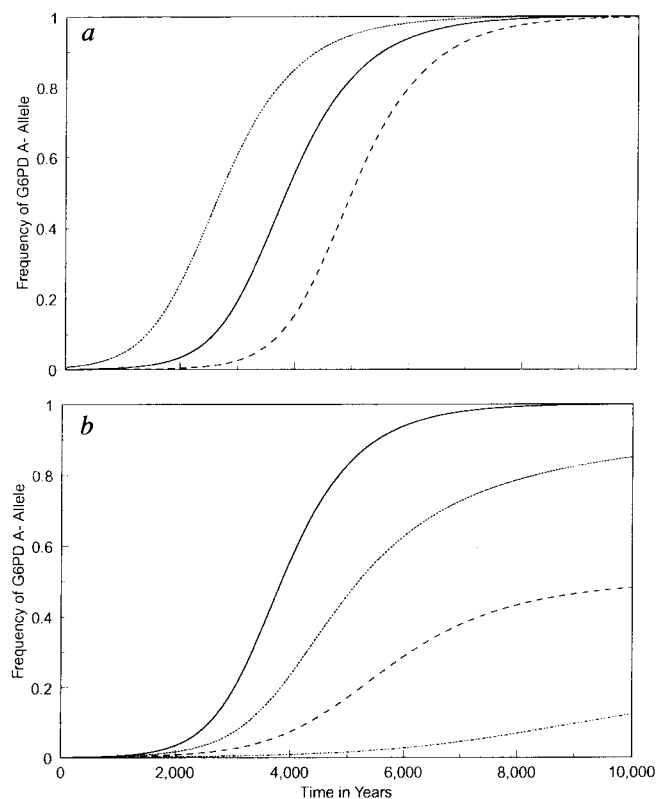
Clearly, the G6PD A- allele is associated with substantial resistance to severe malaria in hemizygous males as well as in heterozygous females. Protection of hemizygotes as well as heterozygotes assists in assessing the proposed mechanisms underlying protection in this disorder and is consistent with *in vitro* studies showing impaired growth of *P. falciparum* in enzyme-deficient erythrocytes²⁴. The degree of protection associated with G6PD deficiency reported here is less than that afforded to carriers of sickle haemoglobin but equal to or greater than that associated with some HLA variants and thalassaemias^{21,23}. Compared with the most prevalent Mediterranean and some Asian types of G6PD deficiency, the African A- variant has a higher level of enzyme activity and the associated disorder is milder¹⁵, so we predict that protection against malaria conferred by G6PD deficiency could be at least as great in many non-African populations.

An important consequence of protection of male hemizygotes as well as female heterozygotes is that this allows a deficiency allele to eventually reach fixation (that is, a frequency of 100%), whereas with heterozygote protection alone the deficiency allele frequency will stabilize at 50%. The availability of data on malaria mortality rates from sub-Saharan Africa²⁵ allows the estimation of the time it would take for the G6PD A- allele to rise in frequency under this degree of positive selection by malaria. Interestingly, the time required is short, approximately 2,000–3,000 years (Fig. 1). Estimates of the time depth of malar-

ial selection in Africa based on archaeology, population history and human genetics are greater: at least 5,000–7,000 years, and possibly much longer²⁶. Furthermore, outside Africa in the many populations where G6PD deficiency is prevalent, deficiency alleles have generally failed to reach frequencies greater than 5–40% (ref. 27). Hence, the magnitude of protection against malaria observed here suggests that a counterbalancing selective disadvantage associated with the deficiency genotypes (see Fig. 1 legend) may have prevented G6PD deficiency from reaching higher frequencies in so many countries.

The main clinical complication associated with G6PD deficiency today is haemolysis after ingestion of oxidant drugs. However, various infections as well as the consumption of fava beans can also lead to haemolysis¹⁵, and, in the past, infections have been more frequent precipitants of severe haemolytic reactions. Additionally, G6PD deficiency is associated with an increased risk of neonatal jaundice, and environmental factors may influence the rate of this complication¹⁵. Individuals with more severe enzyme deficiency appear more susceptible to all of these manifestations, but the estimate of the degree of malaria resistance afforded to female heterozygotes (see Table 1) is little different from that of male hemizygotes. Hence the overall fitness of female heterozygotes is likely to be greater than that of hemizygotes and homozygotes. This situation constitutes a balanced polymorphism²⁸ and would explain the observed rarity of populations in which frequencies of G6PD deficiency are in excess of 50% (refs 15, 27). Thus the magnitude of the protection afforded

FIG. 1 a, Changes in the frequencies of the G6PD A- allele in males and females over time under a single selection pressure with varying initial frequencies of the A- allele of 1% (dotted line), 0.1% (continuous line) and 0.01% (dashed line). The male and female frequencies are effectively superimposed and cannot be distinguished. It can be seen that with a starting frequency of 0.1% (continuous line) the allele reaches the current frequency in The Gambia (of 5.9%, see Table 1) in 2,280 years and nears fixation after 7,000 years. To reach the frequency found in Kilifi, Kenya, requires 2,970 years. In these simulations the protection against death from malaria afforded to G6PD heterozygotes and hemizygotes is equivalent to the reduction in risk of severe malaria associated with these genotypes in the combined analysis shown in Table 1. It is assumed that the protection afforded to female homozygotes against malaria is equivalent to that of male hemizygotes. The malaria mortality rate here is 8%, estimated from contemporary surveys in The Gambia²⁵ and from the local haemoglobin S heterozygote frequency of 15% (ref. 21), which is assumed to be at equilibrium. The other parameter used in the model is the overall population mortality rate, which here corresponds to a mean lifespan of 20 years. A longer mean lifespan would slow the rate of rise of the A- allele. A higher mortality rate from malaria, an expanding population size, a shorter mean lifespan and a higher initial frequency of the A- allele would all shorten the time required to reach present-day frequencies. b, As malaria appears to have been a major selective force in Africa for at least 5,000–7,000 years²⁶, we assessed which alterations in genotype fitness would reduce the rate of increase in frequency of the G6PD A- allele. If the selective advantage to the hemi- and homozygote that is produced by malaria resistance is exactly counterbalanced by increased mortality from other causes, so that overall fitness is equal to that of individuals with a normal G6PD genotype, the rate of rise of allele frequency is reduced and the allele eventually fixes at 50% frequency (broken line). The continuous line is as in a. The dotted line represents a counterbalancing selection pressure corresponding to half the size of the fitness advantage conferred by malaria, and the lowest line shows an overall reduction in fitness of hemizygotes and homozygotes equivalent to the increase in their fitness conferred by malaria resistance, that is, the selective disadvantage is double the size of the selective advantage. If the G6PD A- frequencies observed in Kenya and The Gambia are now at equilibrium, the selective disadvantage to hemizygotes and homozygotes may be calculated to be 1.75 and 2.34 times, respectively, greater than the selective advantage against malaria. The dynamics of this system may be modelled as a set of coupled differential equations $dx_i/dt = \gamma f(i) - \mu(i)x_i$, where the subscript i ($= 1, \dots, 5$) structures the population according to sex and G6PD genotype (1, normal female; 2, heterozygous female; 3, homozygous female; 4, normal male; 5,



hemizygous male), x_i represents the proportion of the total population of genotype i , $\mu(i)$ is the mortality rate associated with genotype i , $f(i)$ is the proportion of the total reproductive output, γ ($= \sum \mu(i)x_i$), assigned to genotype i . If p_r ($= (x_3 + 0.5x_2)/(x_1 + x_2 + x_3)$) and p_m ($= x_5/(x_4 + x_5)$) are the G6PD A- allele frequencies in the females and males respectively, then $f(1) = (1 - p_r)(1 - p_m)/2$; $f(2) = [p_r(1 - p_m) + p_m(1 - p_r)]/2$; $f(3) = p_r p_m/2$; $f(4) = (1 - p_m)/2$; $f(5) = p_m/2$.

against malaria by the African G6PD A- variant suggests that the associated enzyme deficiency disorder, which is relatively benign in a modern medical setting²⁹, may have been selectively much more disadvantageous in the past. □

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A conserved system for dorsal–ventral patterning in insects and vertebrates involving *sog* and *chordin*

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DORSAL–VENTRAL patterning within the ectoderm of the *Drosophila* embryo requires seven zygotic genes, including *short gastrulation* (*sog*)¹. Here we demonstrate that *sog*, which is expressed in the ventrolateral region of the embryo that gives rise to the nerve cord², is functionally homologous to the *chordin* gene of *Xenopus*, which is expressed in the dorsal blastopore lip of the embryo and in dorsal mesoderm, in particular the notochord³. We show by injections of messenger RNA that both *sog* and *chordin* can promote ventral development in *Drosophila*, and that *sog*, like *chordin*³, can promote dorsal development in *Xenopus*. In *Drosophila*, *sog* antagonizes the dorsalizing effects of *decapentaplegic* (*dpp*)^{1,2,4}, a member of the transforming growth factor- β family. One of the *dpp* homologues in vertebrates, *bmp-4*, is expressed ventrally in *Xenopus*⁵ and promotes ventral development^{6,7}. We show that *dpp* can promote ventral fates in *Xenopus*, and that injection of *sog* mRNA counteracts the ventralizing effects of *dpp*. These results suggest the molecular conservation of dorsoventral patterning mechanisms during evolution.

The *sog* protein has 1,038 amino acids and contains four cysteine-rich repeats similar to those in the extracellular proteins thrombospondin, $\alpha 1$ procollagen and von Willebrand factor² (Fig. 1 legend). Loss of *sog* activity results in a partial decrease in ventral neurogenic ectoderm with a corresponding increase in dorsal ectoderm^{1,2,4}. To test the capacity of *sog* to form ectopic ventral structures in wild-type *Drosophila* embryos, 100 pg of synthetic *sog* mRNA was injected into the dorsal region of embryos (Fig. 1). Injection of *sog* mRNA completely ($n=110$) blocked the differentiation of amnioserosal cells, the most dorsal

pattern element (Fig. 1c, d). In a second series of experiments, 47% ($n=274$) of embryos injected with *sog* mRNA differentiated patches of ventral denticles on their dorsal sides (Fig. 1a, b). Many of these injected embryos also lacked dorsally or dorso-laterally derived structures of the head and tail: the anterior maxillary sense organs were missing in 95% of embryos, and the posterior filzkörper were missing in 41% of embryos. Furthermore, the ventralization of the cuticle was reflected in expansion of the underlying central nervous system (CNS): 93% of embryos ($n=86$) that were injected with *sog* mRNA contained dorsal patches of cells that stained with the BP102 monoclonal antibody, which recognizes an epitope in *Drosophila* specific to CNS axons⁸ (Fig. 1e, f). Injection of mRNA encoding a truncated form of the *sog* protein had no phenotypic effect in any assay (Fig. 1 legend). Thus ectopic localization of *sog* mRNA is sufficient to expand the ventrolateral neurogenic ectoderm at the expense of dorsal structures, and to induce the formation of ectopic CNS.

Embryos laid by females homozygous for a null allele of *dorsal*, *dl*¹, lack *dorsal* protein, do not express any ventral-specific genes, and differentiate a cuticle comprised of only the most dorsal pattern elements⁹ (Fig. 1g). Almost all (93%, $n=54$) *dl*¹ embryos injected with 100 pg of *sog* mRNA differentiated dorso-laterally derived structures, such as filzkörper and/or antennal sense organs (not shown), which are never observed in un-injected embryos ($n=104$) or in embryos injected with control mRNA (Fig. 1 legend). Injection of *sog* mRNA at a 7-fold higher concentration caused 70% ($n=20$) of embryos to differentiate a ring of filzkörper around their circumference (Fig. 1h–j). Thus injection of *sog* mRNA transformed *dl*¹ embryos from a dorsalized (D0) phenotype to a dorsolateralized (L2) phenotype¹⁰, indicating that *sog* can promote ventral fates in the absence of *dorsal* gene activity.

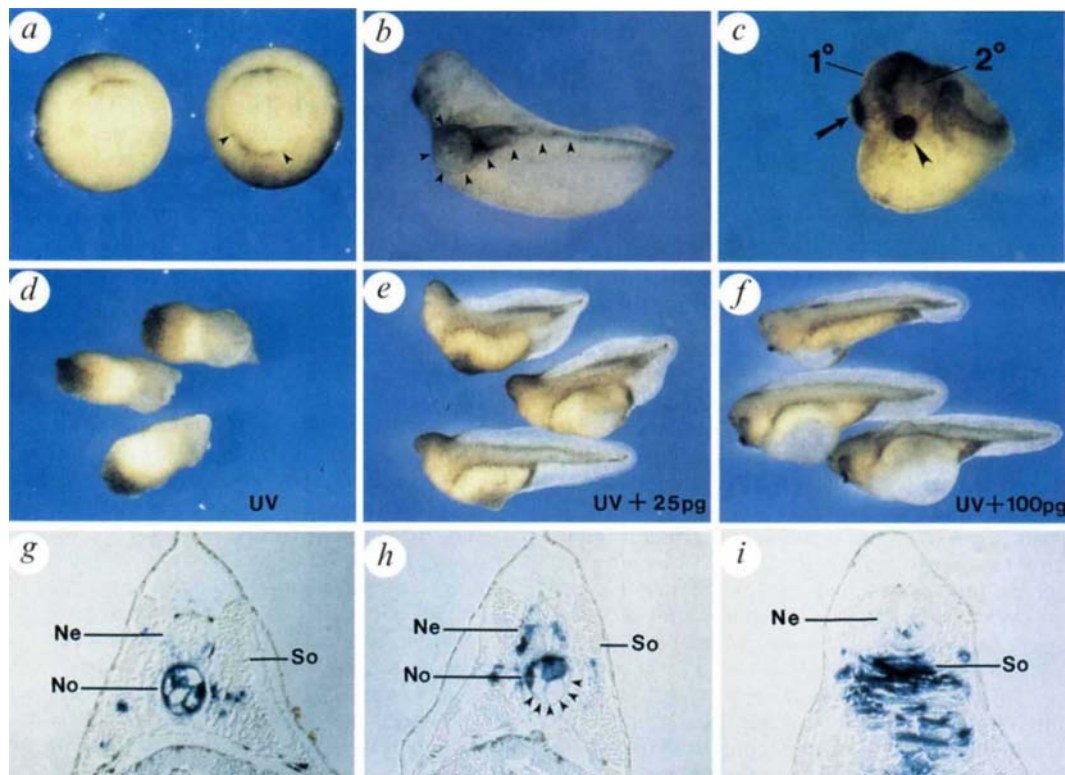
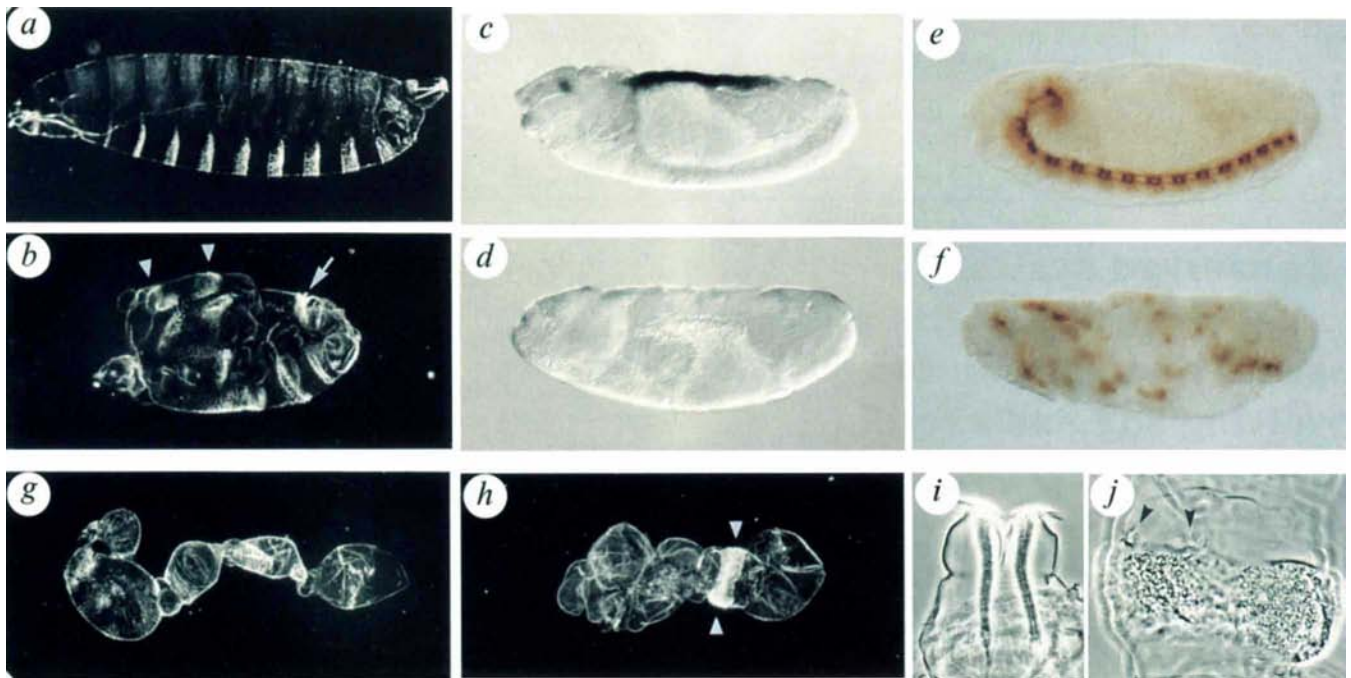
We suggest that *sog* could function by directly blocking the dorsalizing activity of *dpp*, for example by binding and sequestering *dpp* protein. Alternatively, *sog* could encode a growth factor that has activity independent of *dpp*. In wild-type embryos, although *sog* function is not absolutely necessary for the formation of neurogenic ectoderm, ectopic *sog* activity is sufficient to promote formation of this tissue type. We propose that specification of the neurogenic ectoderm requires the action of one or more genes in addition to *sog* that are expressed under *dorsal* control in the ventral regions of the embryo.

The *sog* protein is 28.7% identical to the product of the *chordin* gene in *Xenopus*¹¹ (Fig. 1 legend), which is capable of promoting dorsal fates after mRNA injections into *Xenopus* embryos³. When *sog* mRNA was injected into the ventral marginal region of *Xenopus* embryos at the 4 to 16 cell stage, a second dorsal lip

could be observed in some embryos (Fig. 2a). Injection of *sog* mRNA into single ventral blastomeres resulted in the formation of secondary (twinned) axes (47%, $n=96$, 3 experiments) or in dorsalized embryos (37%). The secondary axes were usually incomplete (Fig. 2b, arrowheads), lacking head structures anterior to the hindbrain. In a few cases (4%) anterior head structures were duplicated (Fig. 2c). Control mRNAs (β -galactosidase or human prolactin) or an RNA encoding the amino terminus of the *chordin* protein had no biological activity in these assays³.

Xenopus embryos irradiated with ultraviolet light before first cleavage form a 'belly piece' consisting entirely of ventral meso-

derm (Fig. 2d). Injection of *sog* mRNA at the 8- or 16-cell stage into a single vegetal blastomere of embryos irradiated with ultraviolet light rescued axial structures in a dose-dependent manner (Fig. 2e,f). Lineage tracing experiments using β -galactosidase mRNA to mark the *sog*-injected cell indicate that the descendants of this cell exhibit multiple properties of the Spemann's organizer. These lineage-traced cells become part of the dorsal axis, preferentially forming notochord and a few cells of the somites (Fig. 2g), as do organizer cells after grafting to host embryos¹². In addition, the lineage-traced cells are able to recruit uninjected cells to form notochord (arrowheads in Fig. 2h), another property of grafted organizer tissue¹³. In embryos



injected with low doses of *sog* mRNA, the notochord does not form, and the lineage-traced cells become muscle cells of the somites in the midline underlying the neural tube (Fig. 2i). Thus *sog* has potent dorsalizing activity in *Xenopus*, promoting formation of organizer-derived tissue, such as notochord and medial somites.

Injection of 800 pg *chordin* mRNA into the dorsal side of *Drosophila* embryos had little effect on embryonic patterning (Fig. 3b and data not shown). One difference between the *chordin* and *sog* proteins is in their N-terminal regions (Fig. 3a): the first 19 amino acids of the *chordin* protein comprise a signal sequence³, whereas amino acids 56 to 74 of the *sog* protein are predicted to form a type-II transmembrane domain². We constructed a plasmid that encoded a chimaeric protein containing the N-terminal region of the *sog* protein fused to the mature *chordin* protein (Fig. 3a). After injection of 200 pg of the chimaeric mRNA into the dorsal side of *Drosophila* embryos, 87% ($n=112$) of injected embryos failed to differentiate amnioserosa (Fig. 3c), and 67% ($n=119$) of cuticles of injected embryos were weakly ventralized (Fig. 3d), as indicated by a loss of dorsal head structures and internalization or fusion of posterior filzkörper. However, the injected mRNA failed to induce ectopic CNS (not shown). Thus, by cuticle morphology and histochemical staining, these injected embryos have phenotypes very similar to partly ventralized embryos mutant for weak *dpp* alleles⁴. Injection of 750 pg of chimaeric mRNA

into embryos from *dl*¹ mutant mothers was sufficient to cause 40% ($n=149$) of embryos to differentiate filzkörper (Fig. 3e). Because injection of control mRNA encoding the N terminus of the *sog* protein lacks biological activity in these assays (Fig. 1 legend), we conclude that *chordin* has ventralizing activity in *Drosophila*, although it is much less active than *sog*. Furthermore, these results suggest that the initial failure of *chordin* mRNA to ventralize *Drosophila* embryos was due to inefficient secretion or improper processing of the *chordin* protein in *Drosophila*.

In *Drosophila*, *sog* antagonizes the dorsalizing activity of *dpp*. To determine whether this interaction could be recapitulated in a heterologous system, *dpp* and *sog* mRNAs were injected into *Xenopus* embryos. The *dpp* homologue in *Xenopus*, *bmp-4*, can inhibit dorsal induction in animal caps caused by activin treatment^{6,7}. A similar effect on activin-treated animal caps was observed after injection of *dpp* mRNA (Fig. 4a–c). Histological sections of injected animal caps revealed the presence of blood cells and other ventral mesodermal tissues (Fig. 4c, insert). In intact embryos, injection of *dpp* mRNA into each animal blastomere at the 8 cell stage completely ventralized the embryo, resulting in a phenotype similar to that observed after irradiation with ultraviolet light (Fig. 4d, e). Injection of *sog* mRNA into one vegetal blastomere of *dpp*-injected embryos resulted in rescue of the ventralized phenotype (Fig. 4f). We conclude from these experiments that *dpp* has ventralizing activity in *Xenopus*, and

◀FIG. 1 Promotion of ventral development in *Drosophila* by *sog*. Lateral view of a wild-type cuticle (a) and a cuticle of a wild-type embryo after dorsal injection of 200 pg *sog* mRNA (b). In the injected embryo, dorsal cells in the anterior abdomen secreted ventral cuticle (arrowheads), and the two posterior filzkörper have fused across the dorsal midline (arrow). Lateral view of a stage-15 wild-type embryo stained for β -gal activity from a P[Kr-lacZ] construct¹⁸ expressed in the amnioserosa (c) and an embryo of identical genotype injected with 100 pg *sog* mRNA in which the amnioserosa failed to develop (d). Lateral view of a stage 14–15 wild-type embryo stained with the CNS-specific monoclonal antibody BP102 (e) and a wild-type embryo stained with the BP102 antibody after dorsal injection with 100 pg *sog* mRNA (f). In the injected embryo the ventral CNS is disrupted and BP102 staining cells are present dorsally. The disruption of ventral CNS in dorsally injected embryos indicates that ectopic localization of *sog* disrupts patterning within the neurogenic ectoderm either as a result of redistribution of a dorsalizing activity (possibly the *dpp* protein), or as a result of alteration in the distribution of a ventral-specific signal (possibly the *sog* protein itself). A cuticle of an embryo laid by a female homozygous for the *dl*¹ mutation (g) and a cuticle of an embryo of identical genotype after injection of 700 pg *sog* mRNA (h). The injected embryo differentiated dorsolaterally derived filzkörper around the embryonic circumference (arrowheads). i, Wild-type filzkörper, the terminal endings of the tracheal trunk. j, The filzkörper of the embryo shown in h. The arrowheads mark two spiracles, protrusions of cuticle associated with filzkörper. METHODS. Differential display¹⁹ was performed on RNA from embryos laid by dorsal females compared with RNA from embryos laid by trunk

cactus females. DNA from a ventral-specific product hybridized to polytene band 13E1–2, the location of *sog*²⁰, and was used to probe a P-element cosmid library²¹. Additionally, genomic DNA adjacent to a P-element integrated into *sog* was used to identify 40 kbp of contiguous DNA from an EMBL 3 phage library²¹. cDNA clones were obtained by screening 0–4 and 4–8 h embryonic libraries²². Integration of DNA from the P-element cosmid S4, which extends 13 kbp upstream and 6 kbp downstream of the *sog* cDNA, rescues *sog* lethality. Two of six nucleotide differences between the *sog* sequence obtained and that reported in ref. 2 cause alterations in the amino-acid sequence of the *sog* protein: changes of A to R at position 980, and F to C at position 1014. The *sog* coding region was amplified by PCR, adding NcoI and SalI sites at the ends, and inserted into pSP35T (ref. 23) to give pSP35T-*sog*. After digestion with SacI, capped mRNA was made by transcribing with SP6 RNA polymerase using the 'Message Machine' kit (Ambion). Control mRNA was made by transcribing pSP35T-*sog* after digestion with BamHI. The control RNA, which encodes the first 356 amino acids of the *sog* protein, had no biological activity in 4 independent assays. First, after injection of 1 ng control RNA, 50% ($n=164$) of the embryos hatched, and none of the remaining embryos had a ventralized cuticle. Second, 99% ($n=170$) of embryos differentiated amnioserosa after injection of 450 pg control RNA. Third, no ($n=119$) embryos displayed ectopic CNS after injection of 450 pg control RNA. Fourth, no ($n=74$) embryos from *dl*¹ mutant mothers differentiated filzkörper after injection of 1 ng of control RNA. RNA injections were performed²⁴ and β -gal activity was assayed²⁴ as described previously.

◀FIG. 2 Secondary axes in *Xenopus* embryos were induced and UV-irradiated embryos were rescued by *sog* mRNA. a, Unlike the control embryo (left), the stage-10 embryo (right) differentiated a second dorsal lip after injection of *sog* mRNA into the ventral side. b, A representative secondary axis induced by *sog* mRNA injection (arrowheads). Histological analysis of sections of this embryo and others ($n=15$) revealed duplication of the auditory vesicle, indicating that the secondary axis extended to the hindbrain. c, Complete twinned axis containing secondary eyes and cement glands (the arrowhead indicates the secondary and the arrow the primary cement gland). d, Embryos completely ventralized by UV light at the one-cell stage. e, Partial rescue of the ventralized phenotype by injection of 25 pg *sog* mRNA. f, Complete rescue of the ventralized phenotype by injection of 100 pg *sog* mRNA. Data in e and f are from four independent experiments. g, Histological section of a UV-irradiated embryo completely rescued after single-cell injection of *sog* and β -gal mRNAs at the 16-cell stage. Descendants of the injected

cell formed notochord (No) and a few isolated cells in the somite (So). Neural tissue (Ne) was recruited from unlabelled cells. h, Rescued embryo in which only part of the notochord is derived from a *sog*-injected cell. Arrowheads indicate uninjected cells recruited to form notochord. i, Section of an incompletely rescued embryo that lacks a notochord but has lineage label in muscle cells of the somite midline, illustrating that neural tube (Ne) can be induced in the absence of notochord.

METHODS. *Xenopus* manipulations were performed as described previously³. For lineage tracing, an improved β -gal synthetic mRNA was prepared by adding a SalI linker to a HindIII-PstI fragment of the expression vector pCH110 (Pharmacia) and subcloning into pBluescript KS⁺ digested by SalI and PstI. This plasmid, pKS- β gal, was linearized with PstI, and transcribed with T3 RNA polymerase. mRNA was injected at a concentration of 20 μ g ml⁻¹.

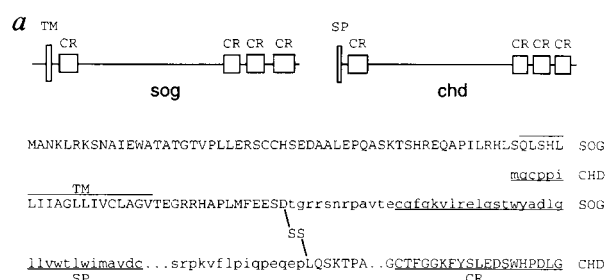


FIG. 3 A chimaeric *sog/chordin* protein partly ventralized *Drosophila* embryos. **a**, Comparison of the *sog* and *chordin* (chd) proteins showing the location of the *sog* transmembrane (TM) domain², the *chordin* signal peptide (SP)³ and the four cysteine-rich repeats (CR). The chimaeric protein (in capital letters) contains the N-terminal 89 amino acids of the *sog* protein, followed by two serine residues, followed by the *chordin* protein starting at amino acid 35. **b**, A stage-15 wild-type embryo carrying the P[Kr-lacZ] construct after injection of 800 pg *chordin* mRNA. Of 83 injected embryos, 65% differentiated amnioserosa. **c**, A stage-15 wild-type embryo carrying the P[Kr-lacZ] construct after injection of 200 pg chimaeric mRNA. The embryo did not differentiate any amnioserosal cells. **d**, A partly ventralized cuticle of a wild-type embryo after dorsal injection of 200 pg chimaeric mRNA, as shown by lack of dorsal head structures and internalization of the filzkörper (arrow). **e**, Filzkörper material differentiated by an embryo laid by a *dl¹* mother after injection of 750 pg chimaeric mRNA. The arrowhead marks a spiracle. **METHODS.** To make the *sog-chordin* chimaera, DNA encoding most of the *chordin* protein was amplified by PCR from pSP35T-chd³ using a primer with *Hind*III and *Xho*I sites followed by sequences encoding amino acids 35 to 42 of the *chordin* protein (5'-GGCAAGCTTGGCTCGAG-CCTCCAATCCAAGACTCCAGCAG-3'). The amplified DNA was digested with *Hind*III and *Xba*I, and subcloned into pSP35T, resulting in pSP35chdΔ #5. DNA encoding the N-terminal region of the *sog* protein was amplified from pSP35T-sog using a primer with sequences encoding amino acids 83 to 89 of the *sog* protein followed by a *Xho*I site (5'-GGCTCTAGAGGCTCGAGTCCGACTCCTCGAACATGAGC-3'). The PCR product was digested with *Hind*III and *Xho*I and ligated into pSPchΔ #5.

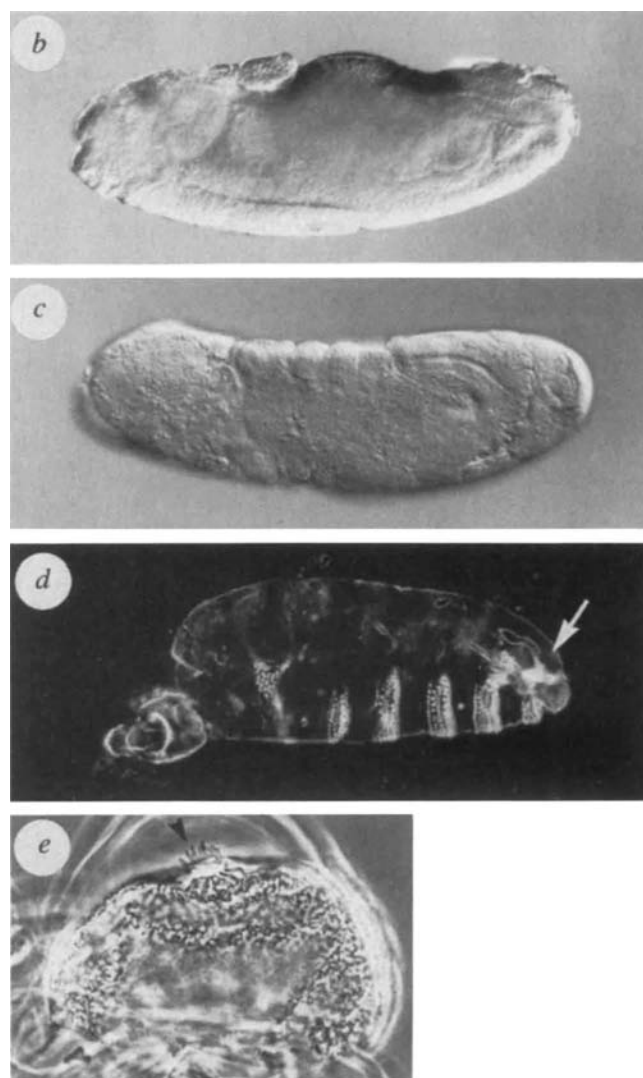
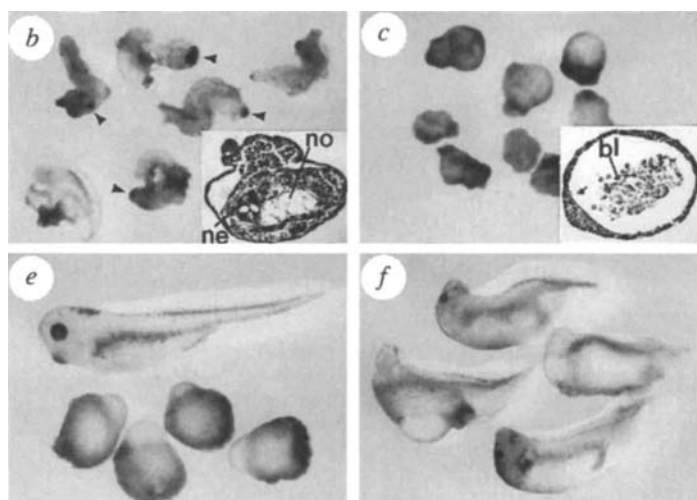
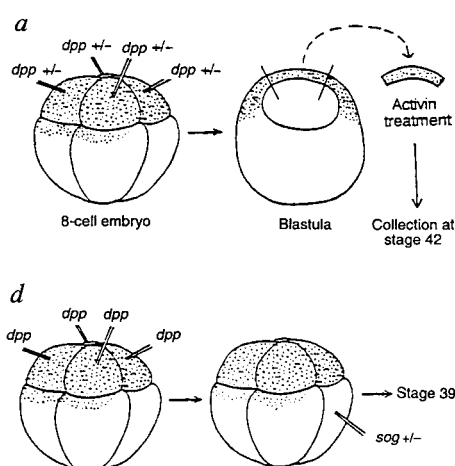


FIG. 4 Promotion of ventral development in *Xenopus* by *dpp*; this effect can be antagonized by *sog*. **a**, Experimental design of animal cap assays. **b**, Animal caps were treated with recombinant human activin (30 ng ml⁻¹ for 2 h; gift from Genentech) and collected at stage 42. The elongation is due to the induction of dorsal mesoderm (muscle and notochord) in these samples. Arrowheads show cement glands, and the insert shows an explant section (no, notochord; ne, neural tissue). **c**, Animal caps from embryos injected radially with *dpp* mRNA (100 pg per animal blastomere at the 8-cell stage) do not elongate after treatment with activin. Histological analysis (insert) revealed ventral mesoderm (containing blood, bl) indicating the *dpp* can completely ventralize mesoderm induced by activin. **d**, Experimental design in which *dpp* mRNA (100 pg) was injected into each of 4 animal cells at the 8-cell stage, and its effect rescued by a subsequent injection of *sog* mRNA



(360 pg) into a vegetal blastomere. **e**, Wild-type *Xenopus* embryo (top) and three embryos completely ventralized by injection of *dpp* mRNA. **f**, *dpp*-ventralized embryos rescued to variable degrees by a single injection of *sog* mRNA into a vegetal blastomere at the 8-cell stage. The extent of rescue was such that a dorso-anterior index of 0.05 ($n=21$) was changed to 2.2 ($n=22$, data from 2 independent experiments). **METHODS.** The *dpp* cDNA expression vector in pGEM-7zf(+)¹⁸ was linearized with *Xho*I and transcribed with SP6 RNA polymerase.

that this activity can be antagonized non-cell autonomously by *sog*.

These experiments demonstrate that dorsal-ventral patterning in both *Drosophila* and *Xenopus* is dependent upon a system involving two extracellular proteins, Dpp/Bmp-4 and Sog/Chordin. Despite fundamental morphological differences between early embryos of the two species, by the late blastula/early gastrula stage the *sog/chordin* gene is expressed in each embryo on the side from which the CNS arises, while the *dpp/Bmp-4* gene is expressed in cells on the opposite side of the embryo. Moreover, the activity of each gene promotes development of the tissue type in which it is expressed. These results support the view that there was a reversal in the dorsal-ventral axis after the divergence of the common ancestor of insects and vertebrates^{14, 17}. □

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Activation of posterior gap gene expression in the *Drosophila* blastoderm

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THE process of body prepattern during *Drosophila* blastoderm formation relies on the localized activities of zygotic segmentation genes, which are controlled by asymmetrically distributed maternal determinants^{1,2}. The anterior determinant bicoid, a homeodomain transcription factor^{3,4}, forms an anterior-to-posterior concentration gradient¹⁻⁴. It interacts with the maternal transcription factor hunchback⁵ to activate the anterior zygotic patterning genes, including the central gap gene *Krüppel* (*Kr*)⁶. In contrast, the posterior maternal system^{1,2} does not provide such a decisive transcription factor, but rather prevents the repressor hunchback from acting in the posterior half so that the gap genes *giant* (*gt*) and *knirps* (*kni*) are activated by an as yet unknown transcription factor^{2,7}. Here we show that caudal, a conserved homeodomain protein that forms a posterior-to-anterior concentration gradient^{8,9}, and the anterior determinant bicoid cooperate to form a partly redundant activator system in the posterior region of the embryo.

In the search for factor(s) that activate posterior *kni* gene expression, we examined deletion constructs of the 2-kilobase (kb) *kni* cis-acting region⁷ for their ability to conduct reporter gene expression in transgenic embryos. Figure 1 shows that gene activation in the *kni*-domain depends on two separate activator modules of 64 bp and 223 bp that respond to the homeodomain transcription-factor gradients provided by the anterior morphogen bicoid^{1,2} and the posterior morphogen caudal (Fig. 1a,

b), respectively. Reporter genes containing the *kni*64 element are expressed in *caudal* mutants but not in embryos lacking *bicoid* activity, whereas expression mediated by the *kni*223 element is affected in *caudal* embryos but not in embryos lacking *bicoid* (Fig. 1c, d). This suggests that caudal regulates transcription of *kni* through the *kni*223 element and bicoid mediates transcription via the *kni*64 element (Fig. 1e).

The *kni*64 and *kni*223 elements are not only necessary (Fig. 1f) but also sufficient to mediate gene activation in response to bicoid and caudal, respectively (Fig. 2a, b). However, the combined *kni*223/*kni*64 element conducts gene activation in the posterior but not in the anterior half (Fig. 2c). This indicates that the *kni*64 element is not able to mediate bicoid-dependent activation in the anterior region of the embryo when combined with the *kni*223 element. In fact, the *kni*223 element contains two binding sites for hunchback (Fig. 1b). As hunchback is a strong *kni* repressor^{2,7}, we deleted these binding sites. The resulting Δ hb-*kni*223/*kni*64 element causes gene activation throughout the embryo (Fig. 2d). This finding suggests that hunchback represses directly through these binding sites and thereby restricts ubiquitous gene activation in response to the combined activities of bicoid and caudal.

The bicoid-responsive *kni*64 element contains six tightly clustered bicoid binding sites (Fig. 3a-c). The expression patterns of reporter genes that contain site-specific deletions and/or combinations of different binding sites for bicoid (Fig. 3f-k) indicate that four sites are sufficient to provide posterior gene expression, and that the ability to activate *kni*-like gene expression is not dependent on specific binding sites for bicoid, their orientation or their affinity (results not shown). Thus their specific arrangement within the element may allow them to sense the low bicoid concentrations in the posterior half of the embryo and to mediate gene activation by a currently unknown mechanism. In contrast, the caudal-responsive *kni*223 element contains six variably spaced binding sites for caudal, of different affinities (Fig. 3d, e). Two of the high-affinity sites are sufficient to mediate caudal-dependent gene activation (Fig. 3l). As caudal does not bind in a cooperative fashion (results not shown), we assume that the *kni*223 element mediates gene activation by the high-affinity binding sites, which respond to a critical threshold value of combined maternal and zygotic activities of caudal^{2,7}.

The phenotypes of embryos lacking caudal and bicoid activities indicate that neither is sufficient to establish a normal ab-

FIG. 1 *kni* cis-acting elements mediating *bicoid*- and *caudal*-dependent activation. **a**, RNA *in situ* hybridization (top) and antibody staining (bottom) showing the expression of the graded maternal (left) and zygotic *caudal* (right). Zygotic *caudal* expression occurs in a broad posterior domain covering the *kni* and *gt* posterior expression domains. Orientation of embryos is anterior left and dorsal up. **b**, Protein binding sites within the 2-kb *kni* control region. Arrow indicates transcription start; the position of the *kni*223 element ('223') and *kni*64 element ('64') are shown by narrow- and wide-striped boxes, respectively. Note that the S/Hc fragment mediates *Kr*-dependent enhancing activity and *til*- and *hb*-dependent repression⁷. RI, *Eco*RI; N, *Nru*I; H, *Hind*III; K, *Kpn*I; S, *Sty*I, C, *Cl*at; Hc, *Hinc*II. **c–f**, Reporter gene constructs lacking the *kni*223 and/or the *kni*64 element and corresponding expression patterns in transgenic embryos (wild-type embryos are to the left, embryos lacking *bicoid* activity are in the middle, embryos lacking both maternal and zygotic *caudal* activity are to the right). **c**, A transgene containing the *kni*223 and the *kni*64 element is expressed in the wild type and in embryos lacking either *bicoid* or *caudal* activity. **d**, A transgene lacking the *kni*223 element is activated in the wild type and in the absence of *caudal*, but not in the absence of *bicoid*, activity. **e**, A transgene lacking the *kni*64 element is activated in the wild type and in the absence of *bicoid*, but not in the absence of *caudal* activity. **f**, A transgene lacking both elements is not activated in wild-type embryos. For the orientation of embryos see **a**. Note that an anterior expression domain (**d**, **e**, left and right photograph) is due to promoter sequences of the reporter gene rather than to *kni* sequences; this domain is variable and appears with a frequency of less than 50% with all constructs analysed. **METHODS**. RNA *in situ* hybridization with β -galactosidase antisense RNA and antibody stainings were performed as described^{5,8}. Antibody-stained embryos (DAPI counterstaining) were examined by laser-scanning microscopy; images were processed by the NIH1.54 software. Transgenic embryos were generated by P-element-mediated transformation²⁴; at least two independent transformant lines were analysed for each experiment shown. Reporter gene expression was examined in various mutant embryos (see below) that were produced by *bcd*^{E1} homozygous females or by *cad*[−] germ-line cloned females. *cad*[−] germ-line chimaeras were generated by use of the yeast recombinase/dominant female sterility (FLP–DFS) technique²⁵, which induces site-specific recombination. Females carrying a *cad*² and FRT recombinant chromosome (genotype: *pr cad*² *P[hs-neo; ry⁺; FRT]^{40A}/CyO*) were crossed to FRT–DFS males (genotype: *P[ry⁺; hs-FLP]¹²; P[w[−]; Ovo^{D121-13x13} P[hs-neo; ry⁺; FRT]^{40A}/CyO*), and the larval progeny heat-shocked at 37 °C. *Cy⁺* progeny were mated to males of the genotype *w; pr cad*²/CyO *P[hb-lacZ]* homozygous for the indicated *kni-lacZ* transgene. Embryos that fail to express the *hb-lacZ* gene allow the distinction between embryos that lack both maternal and zygotic *caudal* activity or maternal *caudal* expression only.

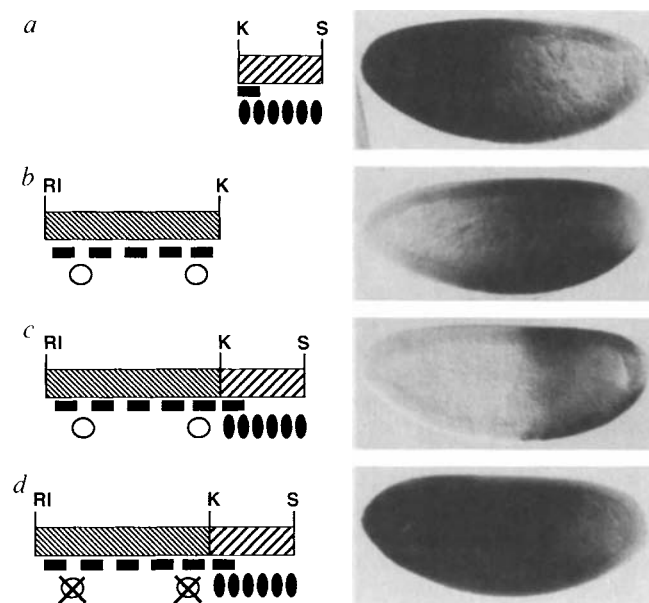
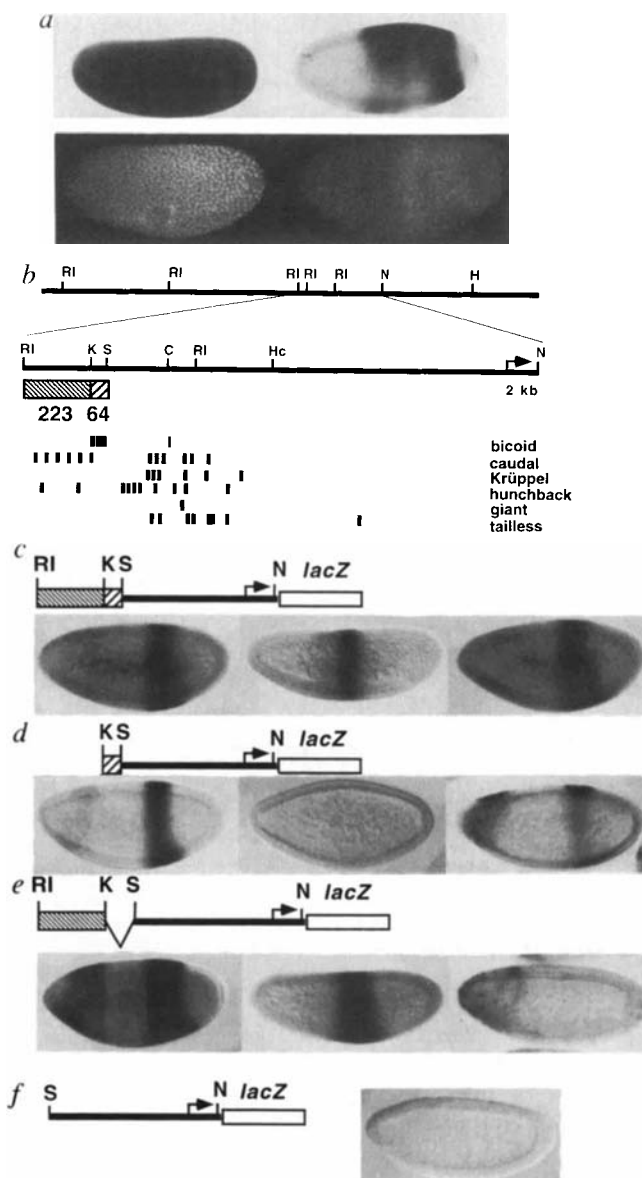


FIG. 2 Combined *bicoid* and *caudal* activities provide activation throughout the embryo. Diagram of *kni*64 and *kni*223 elements (left) conducting reporter gene expression patterns as revealed by *in situ* hybridization (right). Orientation of embryos is anterior left and dorsal up; ovals, rectangles and circles refer to *bicoid*, *caudal* and *hunchback* binding sites, respectively (see Figs 1b and 3). **a**, The *kni*64 element conducts *lacZ* expression in an anterior–posterior gradient in wild-type embryos. No expression was observed in embryos lacking *bicoid* activity (results not shown). Note that the single *caudal* binding site present in the *kni*64 element does not mediate *caudal*-dependent activation (see also Fig. 3h). **b**, The *kni*223 element conducts *lacZ* expression reaching about 60% of egg length (0% is the posterior pole). **c**, The combined *kni*223/*kni*64 element conducts posterior gene expression similarly to that shown in **b**. **d**, The combined *kni*223/*kni*64 element that lacks binding sites for *hunchback* (Δ hb*kni*223/*kni*64 element) provides ubiquitous *lacZ* expression (for details see text). **METHODS**. Reporter gene constructs contain either the *Eco*RI/*Sty*I fragment (the combined *kni*223/*kni*64 element; see Fig. 1), the *Eco*RI/*Asp*718 fragment (*kni*223 element) or *Asp*718/*Sty*I fragment (*kni*64 element) fused to the *hs*43 promoter in front of the *lacZ* gene of the pCaSpeRhs43 P-element vector²⁶. Production of transgenic embryos and *in situ* hybridization are described in Fig. 1. The Δ hb*kni*223/*kni*64 element was generated by polymerase chain reaction *in vitro* mutagenesis; *hunchback* fails to bind *in vitro* whereas the overlapped *caudal* binding site was left unaffected (results not shown).

dominal segmentation pattern. When caudal is absent, the fourth abdominal segment (which is the most sensitive indicator of insufficient *kni* activity¹⁰) is missing and the other abdominal segments are variably affected⁸. In embryos lacking bicoid activity, maternal caudal is present throughout the embryo¹¹. Such embryos develop a normal posterior abdominal segmentation pattern, but their anterior abdominal segment pattern is frequently disturbed¹². These phenotypes are consistent with the argument that in the absence of either bicoid or caudal, posterior segmentation genes are not expressed at biologically relevant levels. In fact, transcription of *kni* and *gt* is altered in embryos lacking caudal activity (Fig. 4). In the absence of bicoid and of zygotic caudal, *kni* is weakly activated and forms a broad domain in the centre of the embryo (Fig. 4c, f), whereas *gt* is not expressed (Fig. 4i, l). In the absence of bicoid and both maternal and zygotic caudal, *kni* is not expressed (results not shown). Furthermore, because caudal functions as a transcriptional activator in transfected tissue culture cells¹³ and is able to activate posterior stripe expression of the pair-rule genes *fushi tarazu* (*ftz*)¹³ and *hairy* (unpublished results), the combined maternal and zygotic caudal activities may function as posterior activator at different levels of the segmentation gene cascade. The partial complementation of the lack of caudal function by bicoid (Fig. 4b, e, h) explains why the major role of caudal as an activator of posterior patterning genes had escaped discovery by formal genetic studies.

Our results establish that posterior gap genes are activated in response to the combined activities of two roughly reciprocal transcription factor gradients to simultaneously blueprint the segment equivalents along the anterior-posterior axis of the *Drosophila* preblastoderm^{1,2} and to generate all segments at the same time during gastrulation. The sequential generation of abdominal segments within an initially uncommitted cellular growth zone of the gastrula¹⁴ of more primitive insects was taken to speculate that the molecular system acting in basic *Drosophila* segmentation may not function within the cellular context^{15,16}. This assumption was supported by the lack of success in identifying *bicoid* homologues in insects other than higher dipterans.

In contrast to *bicoid*, *caudal* homologues are known among various species, and they exert graded expression patterns in a cellular context even in vertebrates¹⁷⁻¹⁹. Furthermore, a *Xenopus* gene with sequence similarity to *nanos*, the decisive component of the maternal posterior system^{1,2} of *Drosophila*, has been isolated²⁰ and *glp*-RNA of the nematode *Caenorhabditis elegans*, which is under translational control by a hypothetical regulator that may be restricted to the posterior cell, has been shown to contain a *nanos*-response element²¹. These observations and the isolation of *hunchback*-like genes in several arthropods²² suggest a conserved model where *nanos* activity eliminates *hunchback* activity from the posterior precellular blastoderm region of more primitive insects; zygotic caudal could then function there at a later cellular stage. In fact, a corresponding cellular gradient

FIG. 3 Molecular organization of the *kni*223 and *kni*64 elements and posterior gene activation in response to *bicoid* and *caudal*. **a-c**, *kni*64 element DNA contains six binding sites for bicoid *in vitro* as revealed by DNase I and hydroxyl radical footprinting (**a**). **A + G**, Maxam-Gilbert reaction; **Free**, control reaction with *kni*64-element DNA; **Bcd**, reaction with *kni*64-element DNA and recombinant bicoid. Bars indicate protected sites. **b**, Bicoid binding sites (see **d**) represent 'weak sites' according to the established consensus²⁷. Underlined sequences represent the core consensus. **c**, Sequence and diagram of binding sites derived from hydroxyl radical footprinting; orientation of binding sites is indicated by arrows. Note that site 4 can be covered in both orientations and that the sites are arranged in three pairs of quasi-palindromic sequences. Contacted nucleotides are 10-11 bp as determined by DMS protection (R.R.-P., unpublished). **d**, The *kni*223 element contains six caudal binding sites of variable affinities as determined by quantitative DNase I footprinting (R.R.-P., results not shown). **e**, Consensus sequences of caudal sites; core is underlined. **f-l**, Reporter gene expression (left) and corresponding transgene constructs (right). Circles: bicoid sites; rectangles: caudal sites. Note that the transgenes shown in **f-k** lack the *kni*223 element but contain either the *kni*64 element (**f**; see also Fig. 1d) or modified *kni*64 elements (**g-k**) in the context of the otherwise intact *kni* cis-acting control region (details in Fig. 1b); modifications are deletions or new combinations of bicoid binding sites (coded by bars according to **c**). Four bicoid sites are sufficient for *bicoid*-dependent gene activation (**g**), whereas two bicoid binding sites are not (**h**). *bicoid*-dependent activation is independent of actual binding site sequences, because a tandem repeat of three copies of the 5' 20-bp sequence of the *kni*64 element (sites 1, 2; see **c**) causes posterior gene expression (**i**) as do five copies of site 5 (**j**). An excess of bicoid sites (five tandem arrays of sites 3-6; see **c**) does not affect the size of the posterior expression domain (**k**). Two high-affinity caudal sites (sites 2, 3) are sufficient for gene activation in the absence of *kni*64 element (**l**). **METHODS**. DNase I²⁸ and hydroxyl radical²⁹ footprinting were performed with a bacterially expressed and purified truncated bicoid (expanding from amino acid 89 to 154) or caudal protein (amino acids 58-377) and the autoradiographs were quantified either by phosphor imaging or densitometry. Other techniques are described in Figs 1 and 2.

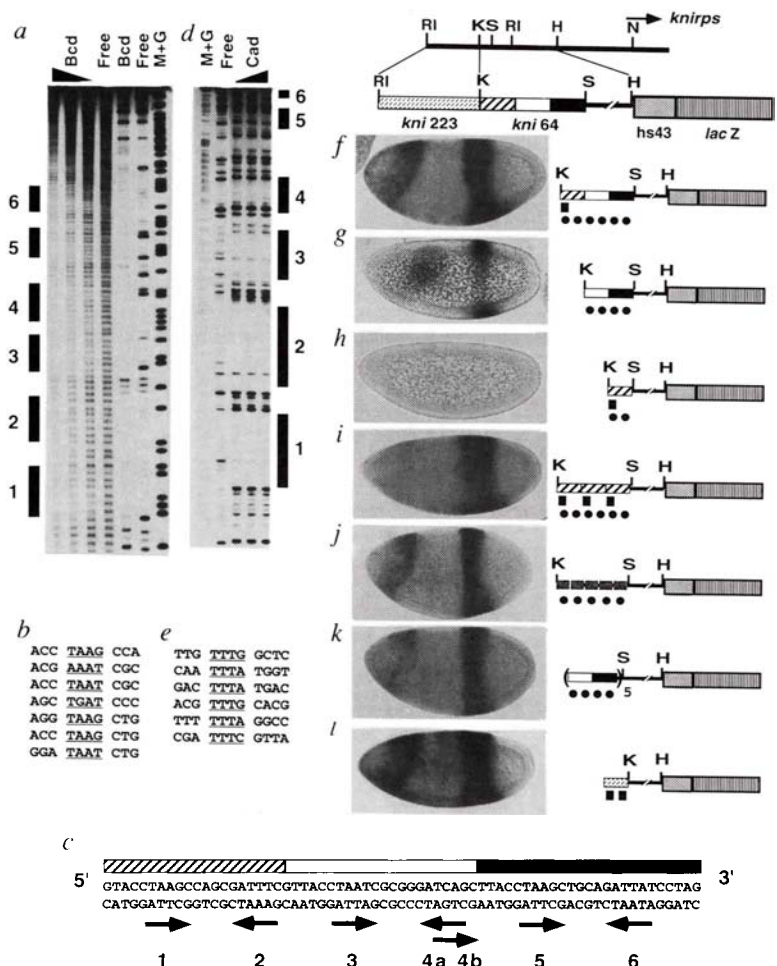
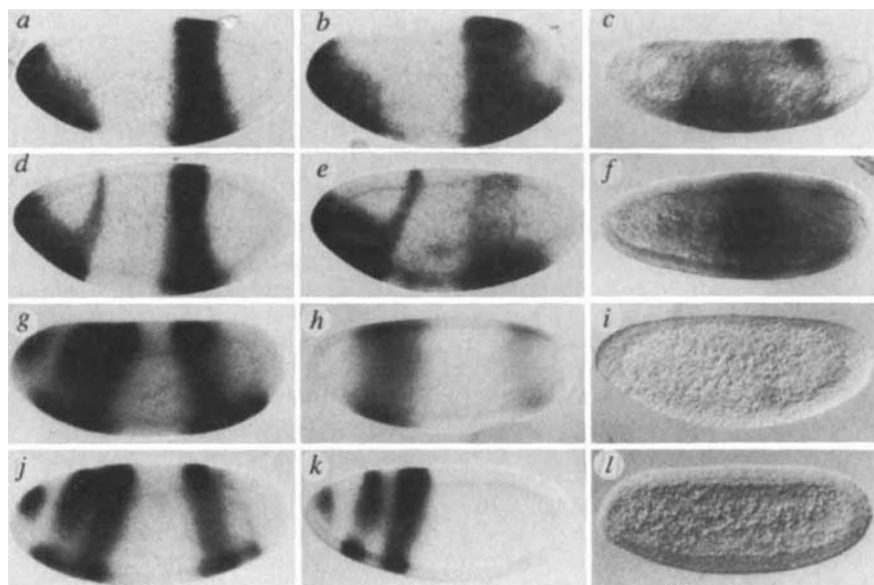


FIG. 4 Posterior gap gene expression directed by bicoid and caudal. *In situ* hybridization showing *kni* (a–f) and *gt* (g–l) expression in wild-type embryos (a, d, g, j), in embryos lacking maternal and zygotic caudal activity (b, e, h, k) and in embryos lacking *bicoid* and zygotic caudal (c, f, i, l). Orientation of embryos is anterior left, dorsal up. In the absence of both maternal and zygotic caudal, *kni* posterior expression is reduced and expands posteriorly at syncytial blastoderm stage (compare a, b and d, e). The posterior expansion of *kni* expression (b, e) might be due to the absence or reduction of a caudal-dependent posterior *kni* repressor such as *gt* (ref. 2) (see below). At cellular blastoderm stage (d, e) both effects are more pronounced. In the absence of *bicoid* and zygotic caudal activity, *kni* is seen only after prolonged staining reactions (c, f); owing to the lack of repressor activities (zygotic hunchback^{5,7} and *gt* (ref. 30); see i, l), weak, *kni* expression covers a broad zone. This indicates that maternal caudal activity activates *kni* only weakly when *bicoid* is absent (c, f). g, h, Reduction of posterior *gt* expression in embryos lacking maternal and zygotic caudal (compare g, h and j, k) is more pronounced than with *kni* (compare b, h and e, k). j, k, The posterior *gt* domain is absent in embryos lacking caudal activity at the cellular blastoderm stage. The low level of initial posterior *gt* expression at syncytial blastoderm stage indicates activa-



tion independent of caudal activity likely to be provided by bicoid (i, l). METHODS. Whole-mount *in situ* hybridization using digoxigenin-labelled *kni* and *gt* antisense RNA and the generation of *cad* mutant embryos and their identification are described in Fig. 1 legend.

of caudal has recently been observed in *Bombyx mori*²³. While forming, the gradient of caudal could provide distinct threshold concentrations to control members of the three-tiered segmentation-gene cascade in space and time, that is, the gradient of caudal could provide the basis for asynchronous segment development in primitive insects by heterochronic gene activation. This role could be analogous to that of the early expression of caudal in vertebrates^{17,19}, and possibly represents a widely conserved feature of early development. Zygotic caudal could therefore represent the ancient function. In this view, its maternal complement and the ability of *bicoid* to support caudal function in the posterior region of the *Drosophila* blastoderm might be a synapomorphy for higher dipterans to adopt a rapid patterning system that acts in a synchronous fashion during early gastrulation. □

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Presynaptic changes during mossy fibre LTP revealed by NMDA receptor-mediated synaptic responses

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ACTIVITY-DEPENDENT changes in synaptic strength are important for learning and memory. Long-term potentiation (LTP) of glutamatergic excitatory synapses following brief repetitive stimulation provides a compelling cellular model for such plasticity^{1–4}. In the CA1 region of the hippocampus, anatomical studies have revealed large numbers of NMDA (N-methyl-D-aspartate) receptor sites at excitatory synapses^{5,6}, which express primarily an NMDA receptor-dependent form of LTP⁷. In contrast, these studies^{5,6} have suggested that mossy fibre synapses activate primarily or exclusively α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and, indeed, these synapses express a form of LTP that is entirely independent of NMDA receptors^{8,9}. Here we present physiological data demonstrating that mossy fibres activate a substantial NMDA receptor synaptic component that expresses

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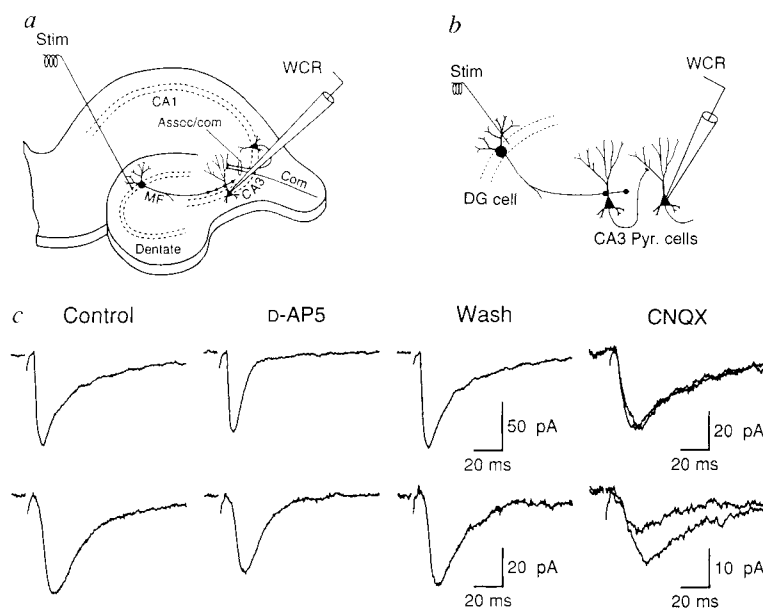
LTP. The presence of an NMDA receptor response allowed us to use the open-channel NMDA receptor antagonist MK-801 to establish directly that the probability of transmitter release is enhanced during the expression of mossy fibre LTP.

Pyramidal cells in the CA3 region receive two anatomically distinct excitatory synaptic inputs: an associational-commissural input, and a mossy fibre input (Fig. 1a). Stimulation in the dentate granule cell layer routinely evoked excitatory postsynaptic currents (e.p.s.cs) recorded in CA3 pyramidal cells that contained a slower 2-amino-5-phosphonovaleate (AP5)-sensitive component (Fig. 1c)^{10,11}. Before we can unambiguously conclude that this NMDA receptor-mediated e.p.s.c. is due to NMDA receptors at mossy fibre synapses, two sources of artefact must be considered. The first is that the NMDA receptor-mediated e.p.s.c. results from the disynaptic activation of associational fibres (Fig. 1b). If the NMDA receptor e.p.s.c. were entirely disynaptic, we would expect that blockade of AMPA receptors would abolish the NMDA receptor e.p.s.c. because, in the absence of AMPA receptors, the mossy fibre synapses would be silent and incapable of activating the disynaptic input. In Fig. 1c (top) the cell was held at -25 mV, and D-AP5 reversibly blocked a slow component of the e.p.s.c. CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) was then added to block the AMPA receptor e.p.s.c. The response recorded in the presence of CNQX and that remaining after subtracting the D-AP5-insensitive component from the control trace were identical, indicating that CNQX had no effect on the NMDA receptor e.p.s.c., which was therefore, along with those from five other cells, entirely monosynaptic. In other cells ($n=5$) CNQX did reduce the NMDA receptor e.p.s.c. (Fig. 1c, bottom). The presence of a disynaptic component seemed to be related to the stimulus strength and, indeed, in two cells the response to low-intensity stimulation was unaffected by CNQX, whereas a CNQX-sensitive component appeared at high stimulus strengths.

The results in Fig. 1 establish that stimulation of the granule cell layer in CNQX can evoke purely monosynaptic responses, but they do not establish that these responses are mediated by mossy fibres. CA3 pyramidal cell collaterals, which form the associational input, also project to the dentate gyrus¹²⁻¹⁴, resulting in the second source of artefact. A stimulating electrode in the granule cell layer could activate these fibres directly, which would monosynaptically activate the associational input via an axon reflex (Fig. 2b). To exclude this possibility we locally applied glutamate, which would be expected to activate granule cells but not fibres, and recorded unitary granule-cell e.p.s.cs in CA3 pyramidal cells. Addition of CNQX and holding the cell at depolarized membrane potentials revealed pure unitary NMDA receptor e.p.s.cs ($n=3$) (Fig. 2a). Thus we have established that mossy fibre synapses directly activate NMDA receptors, a finding in agreement with our preliminary results¹⁵. When using a stimulating electrode in the granule cell layer, however, the potential axon reflex artefact remains. We therefore used a second approach, taking advantage of the finding that in the guinea pig, unlike the rat, mossy fibre responses can be distinguished from non-mossy fibre responses by selective blockade with L-AP4 (refs 16, 17). L-AP4 could markedly reduce the NMDA receptor e.p.s.c. evoked by stimulating at one site in the granule cell layer, but have only a modest effect on the response evoked at another site (Fig. 2c). This result demonstrates that activation of CA3 collaterals can be a major problem. Because of the two forms of contamination discussed above, analysis of mossy fibre NMDA receptor e.p.s.cs required that all experiments be performed in the presence of CNQX, and that only L-AP4-sensitive responses be studied.

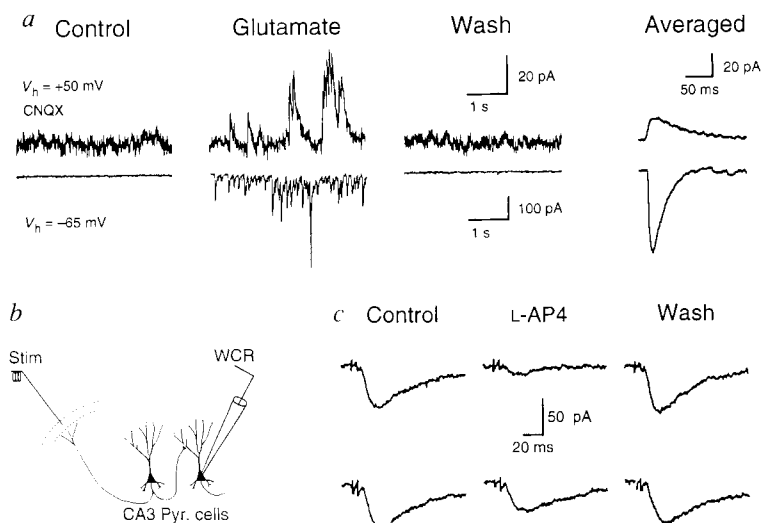
Although selective activation of mossy fibres does evoke an NMDA receptor e.p.s.c., its contribution, relative to the AMPA receptor e.p.s.c., appeared to be considerably less than that found for the associational-commissural e.p.s.c. The ratio of

FIG. 1 Stimulation of the dentate granule cell layer induces monosynaptic NMDA receptor e.p.s.cs. a, Schematic diagram of recording setup in area CA3. E.p.s.cs induced by stimulating the dentate granule cell layer are recorded in whole-cell mode (WCR) from pyramidal cells. These cells also receive synaptic input from associational (Assoc) fibres from ipsilateral CA3 cells and commissural (Com) fibres from contralateral CA3 cells. b, Schematic diagram of the potential disynaptic input to a recorded CA3 pyramidal (Pyr.) cell induced by stimulating dentate granule (DG) cells. c, Top, sample e.p.s.cs recorded at -25 mV holding potential before (Control), during (D-AP5) and after (Wash) the addition of $25 \mu\text{M}$ D-AP5. The response recorded in the presence of $10 \mu\text{M}$ CNQX is then superimposed on the difference between control and D-AP5 responses (CNQX). Note the change of scale. Bottom, sample e.p.s.cs from another cell held at -20 mV in identical conditions. The response in CNQX is smaller than the original D-AP5-sensitive component. Each trace is the average of 10 responses. METHODS. Standard procedures for preparing and maintaining guinea-pig hippocampal slices were used²⁵. Experiments were done and analysed as previously described²⁶. Whole-cell electrodes and standard solutions were as described²⁶. When NMDA e.p.s.cs were examined, the membrane was switched to positive potentials and $100 \mu\text{M}$ picrotoxin was added to the bathing medium. For some experiments a CsF-DIDS internal solution was used to block inhibitory postsynaptic currents internally²⁷; it contained (in mM): 120 CsF, 10 CsCl, 10 Cs-HEPES, 5 EGTA, 1 DIDS. When NMDA receptor e.p.s.cs were examined with the CsF-DIDS filled electrodes, this was done at depolarized, but negative, holding potentials as the cells were unstable at positive potentials. When an associational-commissural input was used, a second stimulating electrode was placed in stratum radiatum to the CA1 side of the recording electrode and far away from stratum lucidum to avoid activating mossy fibres. Baseline



transmission was monitored at 0.05–0.1 Hz. To obtain larger mossy fibre NMDA receptor e.p.s.cs during the MK-801 experiments, paired-pulses at 5 ms intervals were used. LTP was induced with 4 trains of 100 Hz stimulation for 1 s, separated by 20 s and given at baseline stimulus strength. All values are expressed as means $\pm$ s.e.m. Drugs used were CNQX, MK-801 (Research Biochemicals Inc.); D-AP5 (Cambridge Research Biochemicals); L-AP4, DIDS, picrotoxin (Sigma).

FIG. 2 Mossy fibre synapses have functional NMDA receptors. **a**, Records of a whole-cell recording from a CA3 pyramidal cell are shown before (Control), during (Glutamate) and after (Wash) iontophoresis of glutamate (100 mM, pH 8, 200 nA for 30 s, 10 nA retaining current) onto dentate granule cells. Positioning of the iontophoretic pipette was critical, as small displacements of the pipette resulted in the loss of the response. Responses were obtained at a holding potential (V_h) of -65 mV (bottom) and of $+50$ mV in the presence of $10 \mu\text{M}$ CNQX (top). In each case, an average of 10 unitaries is shown on the right (averaged). **b**, Schematic diagram of the potential associational axon reflex input onto a CA3 pyramidal (Pyr.) cell when stimulating the dentate granule cell layer. **c**, Sample NMDA receptor e.p.s.cs recorded from one slice by stimulating in two different places in the dentate granule cell layer (top and bottom) while in the presence of $10 \mu\text{M}$ CNQX and holding at -25 mV membrane potential. The responses before (Control), during (L-AP4) and after (Wash) the addition of $25 \mu\text{M}$ L-AP4 are shown. Each trace is the average of 5 responses.



NMDA receptor to AMPA receptor e.p.s.cs at mossy fibre synapses was found to be $34.5 \pm 5.2\%$ ($n=5$) of that of the associational-commissural input (Fig. 3a). We were unable to evoke LTP of the NMDA receptor e.p.s.c. at the associational-commissural synapses in any cell tested ($n=5$) (Fig. 3b). In striking contrast, subsequent tetanization of the mossy fibres in the same experiment evoked a robust potentiation in every case (Fig. 3b). In addition, the coefficient of variation (c.v.) of NMDA receptor-mediated e.p.s.cs decreased after mossy fibre LTP (to $59 \pm 9\%$, $n=13$), which, based on classical interpretations, is consistent with an increase in transmitter release.

A comparison between the c.v. of AMPA receptor and NMDA receptor e.p.s.cs before and after LTP in the CA1 region has led to the proposal that, in contrast to NMDA receptors, AMPA receptors are either absent or non-functional at a portion of excitatory synapses before LTP¹⁸. In agreement with these

results, the c.v. of the NMDA receptor e.p.s.c. is considerably smaller than that of the AMPA receptor e.p.s.c. at associational-commissural synapses (Fig. 3c, open triangles), which generate NMDA receptor-dependent LTP. However, at mossy fibre synapses, the c.v. of the NMDA receptor e.p.s.c. is actually larger than that of the AMPA receptor e.p.s.c. (Fig. 3c, filled triangles), as well as much larger than the c.v. of the NMDA receptor e.p.s.cs at associational-commissural synapses onto the same cell (associational-commissural, $38 \pm 8\%$ of mossy fibre, $n=5$). Therefore, at these synapses, which express NMDA receptor-independent LTP, the evidence instead suggests that NMDA receptors are either absent or non-functional at some mossy fibre synapses.

The irreversible use-dependent NMDA receptor antagonist MK-801 can be used to monitor the probability of transmitter release at synapses^{19,20}. Repeated activation of synapses in the

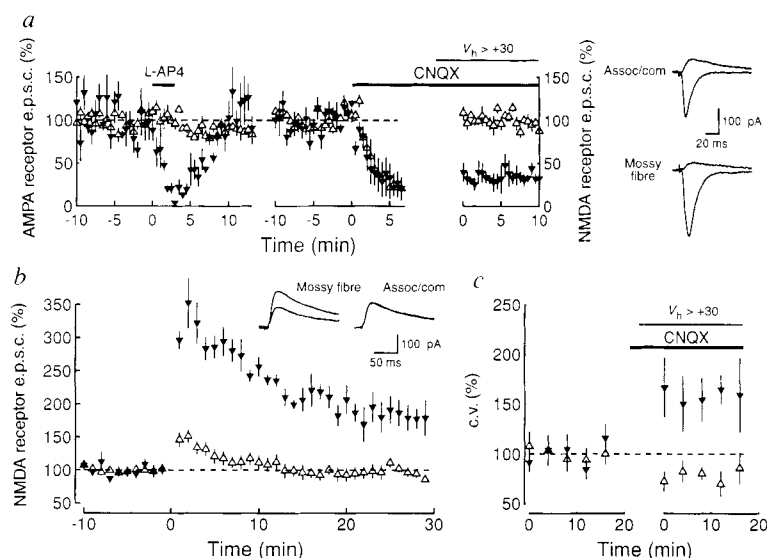
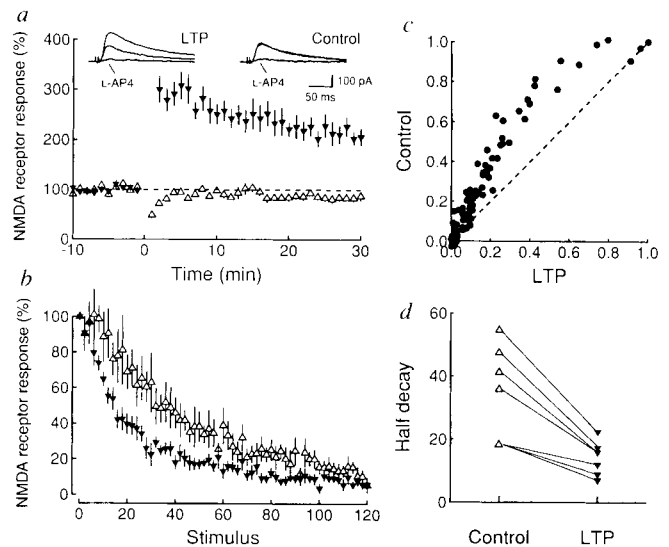


FIG. 3 Comparison of mossy fibre and associational-commissural NMDA and AMPA receptor e.p.s.cs. **a**, Mossy fibre (filled triangles) and associational-commissural (open triangles) e.p.s.cs were recorded in a single cell initially at holding potentials < -65 mV. Application of $25 \mu\text{M}$ L-AP4 (short bar) abolished the mossy fibre responses, but had no effect on the associational-commissural responses. CNQX ($10 \mu\text{M}$, long bar) was then added to the bath and the holding potential switched to $> +30$ mV ($V_h > +30$) to reveal NMDA receptor e.p.s.cs. Once responses had stabilized, they were plotted as a percentage of the associational-commissural NMDA receptor response levels. On average, the mossy fibre NMDA receptor e.p.s.cs were $34.5 \pm 5.2\%$ ($n=5$) of the associational-commissural NMDA receptor e.p.s.cs. **b**, A tetanus given to associational-commissural fibres (open triangles), while holding the cell at positive potentials in the presence of CNQX, failed to induce LTP. In the same cell, subsequent tetanization of the mossy fibres (filled triangles) produced robust LTP ($n=5$). The insets show sample traces from one experiment before and after a tetanus to the associational-commissural fibres and the mossy fibres. Each trace is the average of 10 responses. **c**, The coefficient of variation (c.v.) of AMPA receptor e.p.s.cs was calculated for mossy fibre (filled triangles; $n=5$) and associational-commissural (open triangles; $n=10$) responses at holding potentials < -65 mV. CNQX ($10 \mu\text{M}$, bar) was then added to the bath and the holding potential switched to $> +30$ mV ($V_h > +30$). Once responses had stabilized, the c.v. of NMDA receptor e.p.s.cs was calculated, and is expressed relative to the corresponding AMPA c.v. In every mossy fibre recording, the NMDA c.v. was greater than the AMPA, and the c.v. of the simultaneously recorded associational-commissural responses was the opposite. Values of c.v. are calculated as s.d./mean.

FIG. 4 Mossy fibre LTP is associated with an increase in the probability of transmitter release. **a**, The NMDA receptor-mediated responses of two independent mossy fibre pathways were monitored at positive potentials in the presence of $10 \mu\text{M}$ CNQX ($n=7$; 3 in voltage clamp, 4 in current clamp). At time 0, one was given a tetanus (LTP; filled triangles) during which the other went unstimulated (Control; open triangles). Experiments in which LTP was not stable were discarded. Sample records from one of the experiments are shown above the graph. The records on the left (LTP) show responses recorded in $25 \mu\text{M}$ L-AP4, as well as before and 25 min after a tetanus to this pathway. Responses at the same times in the untetanized pathway are shown on the right (Control). All records are the average of 5 traces. **b**, The stimulation to both pathways was then turned off and $40 \mu\text{M}$ MK-801 was added to the bath. After 10 min, the stimulation was turned on again. The subsequent decay of responses, normalized to the first response after MK-801 addition, was monitored. The responses expressing LTP (filled triangles) decayed more rapidly than the control responses (open triangles). **c**, For each stimulus, the average control and LTP responses are plotted against one another. The general stimulus progression is from point (1, 1) to point (0, 0). **d**, In each individual pathway, the decay of responses was fitted with two exponentials and the number of stimuli necessary to reach the 50% response level (half decay) was determined. The comparison of these values for control (open triangles) and LTP (filled triangles) responses are shown. The corresponding values for individual cells are connected by a line.



presence of MK-801 results in the progressive decline in the amplitude of the NMDA receptor e.p.s.c., as each stimulus releases glutamate and opens NMDA receptor channels, which are then blocked irreversibly by MK-801 and so are unavailable for subsequent responses. The rate of decline of the e.p.s.c. depends on the probability of transmitter release (P_t), because a higher P_t results in more NMDA receptors being opened per stimulus. In a previous study on LTP in the CA1 region²¹ it was found that, although the MK-801 technique provided a sensitive assay for transmitter release, no increase in release could be detected during NMDA receptor-dependent LTP, thus favouring a postsynaptic expression mechanism. There has been disagreement over the interpretation of experiments that used indirect techniques to examine whether the expression of LTP at the mossy fibre synapse is pre- or postsynaptic^{9,22-24}. We therefore used the MK-801 technique to determine if mossy fibre LTP is associated with an increase in transmitter release. Mossy fibre NMDA receptor e.p.s.c.s were evoked by stimulating two independent inputs. LTP was induced in one of the pathways, and a summary graph of all 7 cells is shown in Fig. 4a. After the potentiation had stabilized, the stimulation to both pathways was stopped and MK-801 was added to the perfusate. Stimulation was resumed after 10 minutes. The NMDA receptor e.p.s.c. of the pathway expressing LTP decays considerably faster than that of the control pathway (Fig. 4b). A plot of the size of potentiated responses as a function of the corresponding size of control responses for each time point should describe a straight

line ($y = a + bx + cx^2$; $c=0$) if the decay in both pathways is identical. As is apparent from the graph in Fig. 4c, the deviation from linearity is striking ($c = -1.33$). In every experiment the half decay of the pathway expressing LTP was faster than in the control pathway (potentiated, $45 \pm 4\%$ of control; $P < 0.05$, two-tailed paired t -test) (Fig. 4d).

Our results establish that NMDA receptors are present at mossy fibre synapses, which raises several intriguing questions. Why do mossy fibre synapses not express NMDA receptor-dependent LTP? Might varying the stimulus protocol uncover NMDA receptor-dependent plasticity superimposed on the pre-synaptic NMDA receptor-independent LTP? Alternatively, perhaps the molecular machinery present at synapses that exhibit NMDA receptor-dependent LTP is absent at mossy fibre synapses. The demonstration that mossy fibres activate NMDA receptors has allowed us to gain considerable insight into the mechanisms underlying mossy fibre LTP. The results with MK-801 clearly demonstrate that the probability of transmitter release is enhanced during the expression of mossy fibre LTP. These positive results highlight our previous negative results with MK-801 in the CA1 region²¹, where we were unable to detect any increase in transmitter release during the expression of NMDA receptor-dependent LTP. Our positive results, which suggest a very simple model for mossy fibre LTP in which both the induction and expression are presynaptic, clearly emphasize the fundamental differences in the mechanisms underlying NMDA receptor-dependent and mossy fibre LTP in the hippocampus. □

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A novel receptor involved in T-cell activation

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OPTIMAL T-cell activation and T-cell expansion require triggering by T-cell antigen receptors and co-stimulatory signals provided by accessory cells¹⁻³. A major co-stimulatory pathway involves crosslinking the CD28 molecule on T cells by its ligands CD80 or CD86 expressed on antigen-presenting cells⁴⁻⁷. But recent studies^{8,9} on CD28-deficient mice have indicated that CD28 is not required for all T-cell responses and that additional T-cell co-stimulatory pathways exist. Here we describe a novel glycoprotein, of relative molecular mass 70,000 (*M_r* 70K), designated SLAM, that belongs to the immunoglobulin gene superfamily, which is involved in T-cell stimulation. SLAM is constitutively expressed on peripheral-blood CD45RO^{high} memory T cells, T-cell clones, immature thymocytes, and a proportion of B cells, and is rapidly induced on naive T cells after activation. Engagement of SLAM enhances antigen-specific proliferation and cytokine production by T cells carrying the CD4 antigen (CD4⁺). Particularly, the production of interferon- γ (IFN- γ) is strongly upregulated, even in T helper type 2 (Th2) CD4⁺ T-cell clones, whereas no induction of interleukin(IL)-4 or IL-5 production was observed in Th1 clones. In addition, the engagement of SLAM induces directly the proliferation of CD4⁺ T-cell clones and preactivated T cells, in the absence of any other stimuli, and without CD28 involvement. Thus SLAM is a novel receptor on T cells that, when engaged, potentiates T-cell expansion in a CD28-independent manner and induces a Th0/Th1 cytokine production profile.

We raised monoclonal antibodies (mAbs) to molecules expressed in the early phase of human T-cell activation and identified a mAb (A12) that has unique agonistic effects on T cells and recognizes a previously unidentified early activation molecule, designated SLAM. SLAM is expressed on CD45RO^{high} peripheral-blood memory T cells *in vivo* (Fig. 1). SLAM expression is rapidly induced (within 3 h) on naive CD45RO⁻ T cells and enhanced on CD45RO^{high} T cells after activation, with maximal expression occurring at 6–8 h (Fig. 1). T-cell clones (not shown) and immature CD3^{low}, CD4⁺ and CD8⁺ fetal thymocytes express SLAM constitutively, whereas the more mature CD3^{high} single CD4⁺ or CD8⁺ thymocytes are mostly negative (Fig. 1). SLAM is also expressed on a proportion of freshly isolated peripheral-blood B cells and is upregulated on all B cells after activation (not shown). SLAM was not detected on monocytes (not shown).

SLAM acts as a stimulatory molecule for T-cell activation. The optimal antigen-specific proliferative responses of peripheral-blood T cells of donors immunized with purified protein derivative (PPD) (Fig. 2a) or tetanus toxoid (not shown) were enhanced in a dose-dependent manner to a maximum of 2–3-fold by the addition of anti-SLAM mAb A12 F(ab')₂ (Fig. 2a). The A12 mAb, in the absence of antigen, had no direct mitogenic effects on freshly isolated, resting peripheral-blood mononuclear cells (not shown). Antigen-specific proliferation of CD4⁺ T-cell clones was also enhanced by the A12 mAb or A12 F(ab')₂ fragments in a dose-dependent manner. This enhancement was observed with CD4⁺ T-cell clones belonging to the Th1 (Fig. 2b), Th0 and Th2 (not shown) subsets. The proliferation-enhancing effects mediated through SLAM on T cells were not restricted

to antigen-specific stimulation, as T-cell proliferation induced by anti-CD3 mAbs was also enhanced by A12 (Fig. 2c). Even at supra-optimal anti-CD3 mAb concentrations, a further 2–3-fold increase in proliferation was observed on engagement of SLAM by the A12 mAb.

In addition to its enhancing effects on antigen- and anti-CD3-driven T-cell proliferation, the anti-SLAM mAb A12 or its F(ab')₂ directly induced the proliferation of T-cell clones and pre-activated T cells, but not of freshly isolated resting T cells.

CD4⁺ T-cell clones belonging to the Th0, Th1 and Th2 subsets proliferated vigorously after engagement of SLAM by A12 mAb or its F(ab')₂, in the absence of antigen or any other exogenous stimuli (Fig. 2d, and results not shown). Similarly, peripheral blood mononuclear cells from PPD-sensitized donors, pre-activated with specific antigen or phytohaemagglutinin (PHA), were also induced to proliferate by A12 mAb (Fig. 2e, f). Thus the engagement of SLAM results directly in T-cell proliferation, once the T cells are in an activated state. Direct induction of proliferation of the T-cell clones or preactivated peripheral-blood T cells did not require crosslinking of SLAM, because A12 Fab fragments were also directly mitogenic (not shown). In contrast, engagement of CD28 by anti-CD28 mAb alone did not result in proliferation of the T-cell clones (Fig. 2d), distinguishing SLAM-mediated T-cell activation from that mediated by CD28. Anti-CD86 and anti-CD80 mAbs were unable to block A12-induced T-cell clone proliferation (not shown) although CD80 and CD86 are expressed on T-cell clones and activated T cells^{6,10}, confirming that SLAM acts independently of CD28. The observation that engagement of SLAM directly induces the proliferation of pre-activated T cells indicates that this molecule and its putative ligand play an important role in the expansion of activated T cells.

Cytokine production by a group of CD4⁺ T-cell clones belonging to different T-helper subsets stimulated by their respective antigens was upregulated after SLAM engagement by A12 mAb. In particular, IFN- γ production was strongly enhanced (Fig. 3). Antigen-specific stimulation of Th0, Th1 and Th2 clones in the presence of A12 mAb or its F(ab')₂ resulted in a strong upregulation (5–17-fold) of IFN- γ production. Inter-

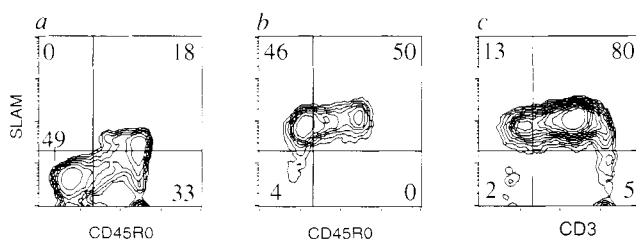


FIG. 1 Cellular expression of SLAM. Surface expression of SLAM on T cells (a, b) and fetal thymocytes (c). Unstimulated peripheral-blood T cells (a) and activated T cells (b) (gated on CD3⁺ lymphocytes), stained with mAbs to CD45RO (abscissa) and SLAM (ordinate). c, Fetal thymocytes stained with anti-CD3 (abscissa) and A12 mAb (ordinate). The numbers in the quadrants indicate the percentages of cells in that particular region.

METHODS. The anti-SLAM monoclonal antibody A12 (IgG1) was generated in a fusion of splenocytes from a BALB/c mouse immunized with peripheral blood mononuclear cells (PBMCs) activated for 5 h with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Calbiochem-Behring, La Jolla) (1 ng ml⁻¹) and the Ca²⁺ ionophore A23187 (500 ng ml⁻¹) (Calbiochem-Behring). PBMCs from a healthy donor were incubated for 6 h with or without TPA and A23187 and stained with cychrome-conjugated anti-CD3 (Pharmingen, San Diego), phycoerythrin(PE)-conjugated A12 mAb and fluorescein-isothiocyanate(FITC)-conjugated anti-CD45RO (Pharmingen). Fetal thymocytes were stained for 30 min with PE-conjugated A12 mAb and FITC-conjugated anti-CD3 (Becton Dickinson, San José, CA). The cells were analysed by flow cytometry using a FACScan (Becton Dickinson). The quadrants used in the analyses were set by gating >99% of PBMC stained with control IgG1-FITC and PE-conjugated mAb (Becton Dickinson) in the lower left quadrant.

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estingly, the levels of IFN- γ produced by Th2 clones in the presence of A12 mAb were comparable to those induced by antigen in Th1 and Th0 clones (Fig. 3). In contrast to its strong IFN- γ -inducing effects on Th2 clones, the A12 mAb did not induce IL-4 (Fig. 3) or IL-5 production by Th1 clones, although SLAM surface expression was comparable in the two subsets (not shown). Furthermore, ligation of SLAM had little (less than twofold) or no enhancing effects on IL-4 production by

antigen-activated Th2 and Th0 clones (Fig. 3, and results not shown). These results indicate that engagement of SLAM during antigen-specific T-cell stimulation results in a preferential induction of IFN- γ production, even in allergen-specific CD4⁺ T-cell clones of the Th2 subset, thereby reversing the phenotype of these cells to a Th0 cytokine production profile. But the cytokine production pattern defining Th1 clones is not altered by concomitant stimulation via SLAM.

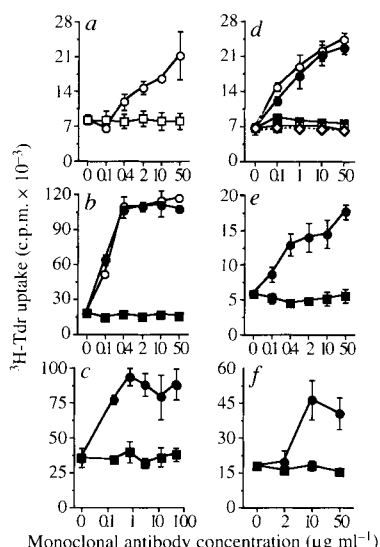


FIG. 2 Effects of mAb A12 or A12 F(ab')₂ on T-cell proliferation. **a**, Proliferation of PBMCs from a purified protein derivative (PPD)-immunized donor stimulated with specific antigen in the presence of A12 F(ab')₂ (open circles) or IgG1 isotype control F(ab')₂ (open squares). **b**, Proliferation of the Th1 T-cell clone HY06 stimulated with the heat-shock protein-derived peptide 2-12 (1 µg ml⁻¹) presented by autologous Epstein-Barr virus (EBV)-transformed B cells in the presence of mAb A12 (filled circles), A12 F(ab')₂ (open circles) or IgG1 isotype control mAb (filled squares). **c**, Proliferation of the T-cell clone B21 (Th0) stimulated with the anti-CD3 mAb spv-T3b (10 ng ml⁻¹) in the presence of mAb A12 (filled circles) or IgG1 isotype control mAb (filled squares). **d**, Proliferation of the T-cell clone ChT38 (Th0) in response to A12 mAb (filled circles), IgG1 isotype control mAb (filled squares), A12 F(ab')₂ (open circles), isotype control IgG1 F(ab')₂ (open squares) or the anti-CD28 mAb L293 (open diamonds). **e**, Proliferation of PBMCs from a sensitized donor activated with PPD for 2 weeks and recultured with mAb A12 (filled

circles) or IgG1 isotype control mAb (filled squares). **f**, Proliferation of PBMCs from a healthy donor activated with PHA for 1 week and recultured with mAb A12 (filled circles) or IgG1 isotype control mAb (filled squares).

METHODS. PBMCs (10⁵ per well) from PPD-sensitized donors were stimulated with 1 µg ml⁻¹ PPD (a kind gift from Å. Andersen) in the presence of A12 F(ab')₂ or IgG1 isotype control F(ab')₂ in flat-bottomed 96-well plates in 200 µl Yssel's medium in triplicate wells¹⁵. The following CD4⁺ T-cell clones were used in the proliferation assays: (i) Th0 clones, B21 specific for tetanus toxoid¹⁶, ChT38 and MoT81 specific for the tetanus toxoid-derived peptide 16-35; (ii) Th1 clones, HY06 (ref. 17) specific for the peptide 2-12 derived from heat-shock protein and TA20 specific for PPD; (iii) Th2 clones, NP12 and NP44 specific for the peptide 94-104 derived from the house-dust-mite allergen Der-p1 (ref. 18). All T-cell clones were harvested 5-7 days after restimulation with irradiated feeder-cell mixture and PHA (Wellcome, Beckenham, UK) as described^{17,18}, washed and cultured in Yssel's medium (5 × 10⁴ per well) in the presence of specific antigen or peptide (1 µg ml⁻¹), and 2.5 × 10⁵ autologous irradiated (5,000 rad) EBV-transformed B cells per well and mAbs as indicated. For the direct induction of T-cell proliferation by the anti-SLAM mAb A12, 5 × 10⁴ cells per well were incubated in 200 µl Yssel's medium with A12 or IgG1 isotype control mAbs or their F(ab')₂ as indicated. For preactivation of T cells by PPD or PHA, PBMCs of PPD-sensitized and normal donors, respectively, were cultured at 10⁶ cells per ml in Yssel's medium in T75 flasks (Falcon, Becton Dickinson) with PPD (1 µg ml⁻¹) or PHA (1 µg ml⁻¹) for 14 to 7 days respectively. At the end of the culture period, the cells were washed in PBS, resuspended in Yssel's medium and cultured (10⁵ cells per well, 200 µl total volume) in round-bottomed 96-well plates in triplicate wells in the presence of A12 mAb or IgG1 isotype control mAb. The cultures were harvested after 3 days (T-cell clones and PHA-stimulated cells) or after 5 days (PPD-activated cells). 1 µCi of ³H-thymidine (Tdr) (Amersham, Arlington Heights, IL) was added to each well during the last 16 h of culture, and proliferation was measured by ³H-Tdr uptake, using a β-counter. All mAbs used in the functional studies were purified from ascites by caprylic acid fractionation¹⁹ followed by ammonium sulphate precipitation. F(ab')₂ were produced by standard methods, digesting the mAbs with pepsin (Sigma, San Louis) then separating the F(ab')₂ fragments by Sephadex gel size fractionation. The isotype control mAb used was IgG1 from MOPC-21.

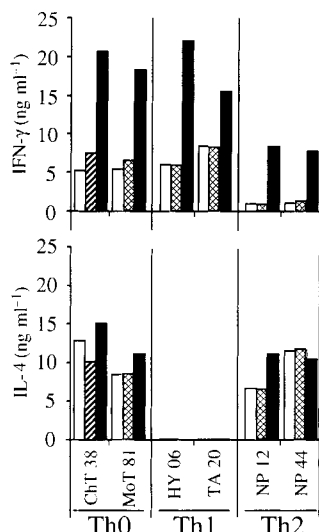


FIG. 3 Cytokine production by T-cell clones stimulated with their specific antigen in the presence of the anti-SLAM mAb A12. Open columns, no antibody; shaded columns, control antibody; filled columns, A12 mAb. **METHODS.** 10⁶ CD4⁺ T-cell clones described in the legend of Fig. 2 were activated by antigen or antigenic peptides, presented by 10⁶ irradiated (5,000 rad) autologous EBV-transformed B cells, in the presence of A12 mAb (10 µg ml⁻¹), or IgG1 isotype control mAb (10 µg ml⁻¹), in a final volume of 1 ml Yssel's medium in 24-well plates (Falcon, Becton Dickinson). The supernatants were harvested 24 h later and the cytokine content was determined by enzyme-linked immunosorbent assay as described^{20,21}.

FIG. 4 Cloning and molecular characterization of SLAM. **a**, Predicted amino-acid sequence of SLAM cDNA. The underlined sequence represents the leader sequence. The transmembrane region is boxed. Cysteines are marked by filled circles and potential N-linked glycosylation sites are boxed in grey. The 1,860 nucleotide SLAM cDNA (pSURslam1) was isolated by expression cloning using the A12 mAb for selection of COS-7 cells transfected with a cDNA expression library, prepared from polyadenylated mRNA purified from an activated T-cell clone²² that expressed SLAM. The two solid arrows indicate the location of the in-frame deletion (encompassing amino acids 234–263) in an alternatively spliced 4-kb cDNA encoding the secreted form of SLAM (pSECslam). The open arrow indicates the position after which the sequence of a SLAM variant (pSURslam2) with a shorter cytoplasmic tail differs, the alternate amino-acid sequence being, in single-letter code, DTHHQTSDLF. The 4-kb cDNA clone (pSECslam) and the 2-kb clone (pSURslam2) were cloned from the same cDNA library described above by standard methods²³, using the insert in pSURslam1 as a hybridization probe. **b**, SLAM immunoprecipitation from radiolabelled B21 T-cell clone lysate. Lane 1, SLAM immunoprecipitate; lane 2, SLAM immunoprecipitate treated overnight with 10 U ml⁻¹ N-glycanase (Genzyme); lane 3, buffer; lane 4, control antiserum precipitate; lane 5, ¹⁴C-methylated molecular mass markers (*M*, 30K–200K) (Amersham). **c**, Distribution of SLAM transcripts. RNA from various cells and tissues was subjected to reverse transcription and PCR using SLAM-specific primers (upper panel) or HPRT (hypoxanthine phosphoribosyltransferase)-specific primers (lower panel): lane 1, JY EBV-transformed B cells; lane 2, CD20⁺ B cells purified from spleen; lane 3, CD8⁺ T-cell clone S11; lane 4, CD8⁺ T-cell clone S40; lane 5, CD4⁺ T-cell clone B21; lane 6, CD4⁺ T-cell clone B21 activated; lane 7, CD56⁺ cells purified from PBMCs; lane 8, CD56⁺ cells purified from PBMCs; lane 9, fetal liver; lane 10, fetal bone marrow; lane 11, fetal thymus; lane 12, small intestine; lane 13, brain; lane 14, kidney; lane 15, heart; lane 16, H1 CD4⁺ T-cell clone; lane 17, FL508 pre-T-cell line; lane 18, TN92 pre-T-cell line; lane 19, H₂O. **d**, Induction of SLAM mRNA. Peripheral blood lymphocytes were incubated with anti-CD3 mAbs (1 µg ml⁻¹) for the times indicated. RNA was extracted and subjected to northern analysis using SLAM and actin probes successively.

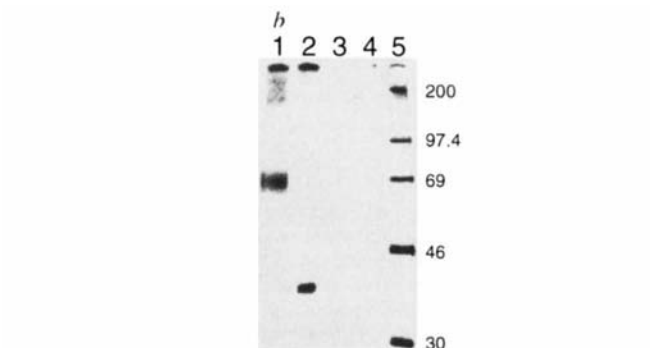
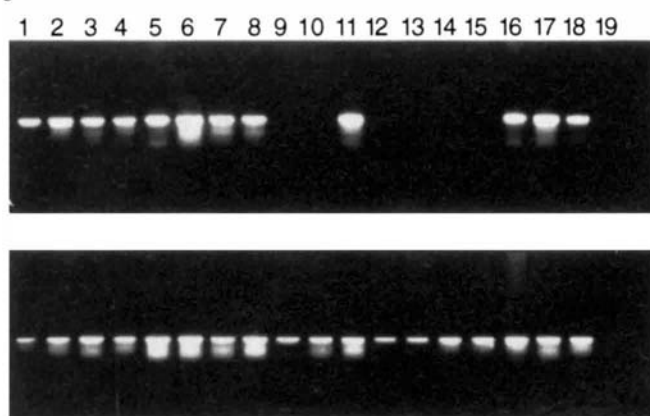
METHODS. COS-7 cells were transfected by electroporation with the A10 CD8⁺ T-cell library cDNA prepared as described²². Transfected cells were stained with FITC-conjugated anti-SLAM mAb A12 and sorted with a FACStar plus (Becton Dickinson). Plasmid DNA was isolated from sorted cells using a Wizard miniprep kit (Promega Corporation, Madison, WI). Plasmid DNA was transformed in *Escherichia coli* (ElectroMAX, BRL, Gaithersburg, MD) by electroporation for amplification and then introduced into COS-7 cells. After two rounds of sorting, SLAM cDNA clones were enriched to 45% of the total cDNA clones. The 1.9-kb insert in one of these clones (pSURslam1) was sequenced in both strands using the dideoxy chain-termination method Sequenase kit (USB, Cleveland, OH). L cells stably transfected, as described²², with pSURslam1 were used to immunize C3H mice to obtain antiserum to recombinant human SLAM. Cell-surface proteins of the Th0 T-cell clone B21 (1.3×10^6 cells) were radiolabelled with ¹²⁵I (as the sodium salt) (Amersham) using the lactoperoxidase-catalysed reaction, and SLAM immunoprecipitation was done as described²⁴, using Pansorbin (Calbiochem) coated with rabbit anti-mouse immunoglobulin and the anti-SLAM antiserum described above. The immunoprecipitates were run on a 10% polyacrylamide minigel (ISS, Natick, MA) under reducing conditions, and the dried gel was scanned and analysed with a Phosphorimager (Molecular Dynamics, Menlo Park, CA). The northern analysis was performed as described²² using the probe described in *a* for SLAM and an actin-specific probe. For PCR analysis the primers were 5'-ATCACTGGAGAAGTGT-3' and 5'-CCCAGCATACACTGCC-3' for SLAM and the previously described primers for HPRT (ref. 22). 5 ng of the cDNAs indicated was subjected to 30 cycles of PCR as described²².

a

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1
met asp pro lys gly leu leu ser leu thr phe val leu phe leu ser leu ala phe gly
21
ala ser tyr gly thr gly gly arg met met asn cys pro lys ile leu arg gln leu gly
41
ser lys val leu leu pro leu thr tyr glu arg ile [asn]lys ser met [asn]lys ser ile
61
his ile val val thr met ala lys ser leu glu asn ser val glu asn lys ile val ser
81
leu asp pro ser glu ala gly pro pro arg tyr leu gly asp arg tyr lys phe tyr leu
101
glu [asn]leu thr leu gly ile arg glu ser arg lys glu asp glu gly trp tyr leu met
121
thr leu glu lys [asn]val ser val gln arg phe cys leu gln leu arg leu tyr glu gln
141
val ser thr pro glu ile lys val leu [asn]lys thr gln glu [asn]gly thr cys thr leu
161
ile leu gly cys thr val glu lys gly asp his val ala tyr ser trp ser glu lys ala
181
gly thr his pro leu asn pro ala [asn]ser ser his leu leu ser leu thr leu gly pro
201
gln his ala asp asn ile tyr ile cys thr val ser asn pro ile ser [asn]asn ser gln
221
thr phe ser pro trp pro gly cys arg thr asp pro ser glu thr lys [pro trp ala val]
241
[tyr ala gly leu leu gly gly val ile met ile leu ile met val val ile leu]gln leu
261
arg arg arg gly lys thr asn his tyr gln thr thr val glu lys lys ser leu thr ile
281
tyr ala gln val gln lys pro gly pro leu gln lys lys leu asp ser phe pro ala gln
301
asp pro cys thr thr ile tyr val ala ala thr glu pro val pro glu ser val gln glu
321
thr asn ser ile thr val tyr ala ser val thr leu pro glu ser

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**c****d**

A complementary DNA encoding SLAM was isolated from a T-cell cDNA library by expression cloning, with A12 mAb for selection. The SLAM cDNA was 1,860 base pairs in length and contained one large open reading frame encoding a type I transmembrane protein with a 27-amino-acid amino-terminal hydrophobic leader sequence, a 202-amino-acid extracellular region that contains 8 potential *N*-glycosylation sites, a 22-amino-acid hydrophobic membrane-spanning portion, and a 77-amino-acid cytoplasmic domain (Fig. 4a). Three of the four potential Tyr phosphorylation sites in the cytoplasmic domain of SLAM conform to the consensus sequence phosphotyrosine-hydrophobic-X-hydrophobic, determined for binding to one class of SH2 domains¹¹. Antisera raised against recombinant SLAM precipitated an M_r 70K glycoprotein from a CD4⁺ T-cell clone (Fig. 4b). *N*-glycanase treatment of the SLAM immunoprecipitate revealed a protein core of M_r 40K (Fig. 4b), which correlates with the molecular size predicted by the cDNA sequence. SLAM is a member of the immunoglobulin supergene family, with one variable and one constant domain, and shows a limited degree of homology with CD48 (ref. 12) (26%), LFA-3/CD58 (ref. 13) (17%) and 2B4 (28%), a recently cloned signalling molecule expressed on murine NK and cytotoxic T cells¹⁴.

The distribution of SLAM was further analysed by using the polymerase chain reaction (PCR) to detect transcripts in various tissues and cell types. SLAM is expressed only in lymphoid cells (Fig. 4c). Activated peripheral-blood mononuclear cells contain a 1.9-kilobase (kb) transcript (Fig. 4d), corresponding to the size of the cloned SLAM cDNA (Fig. 4a), and also a 4-kb transcript. The 4-kb messenger RNA is composed of at least two different transcripts, including one encoding a secreted form of SLAM lacking 30 amino acids encompassing the entire 22-amino-acid transmembrane region (Fig. 4a), and another that encodes the transmembrane form of SLAM described above. An alternatively spliced 2-kb cDNA clone was also identified, encoding a form of SLAM with a truncated cytoplasmic domain (Fig. 4a). SLAM mRNA is induced within 2 h after activation (Fig. 4d), which correlates with its rapid appearance on the T-cell surface (Fig. 1).

In conclusion, our data indicate that SLAM acts as a stimulatory molecule for human peripheral blood T cells. In addition, the engagement of SLAM results in direct T-cell proliferation, once the T cells are in an activated state. The finding that concomitant stimulation through SLAM induces Th0/Th1 cytokine production profiles in antigen-activated T-cell clones, including Th2 clones, suggests that interactions of SLAM with its putative ligand contribute to T-cell expansion and the generation of Th0 or Th1 responses. Finally, the presence of SLAM on thymocytes and B cells, and the natural occurrence of a secreted form of SLAM, indicate a broader function of this molecule than currently described. □

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Failure of B-cell differentiation in mice lacking the transcription factor EBF

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EARLY B-cell factor (EBF) is a cell type-specific transcription factor that is expressed at all antigen-independent stages of B-lymphocyte differentiation and participates in the regulation of the *mb-1* gene^{1–4}. Here we show, by targeted gene disruption in mice, that *EBF* is necessary for the generation of immunoglobulin-expressing B cells. *EBF*-deficient mice lack B cells that have rearranged their immunoglobulin D and J_H gene segments, but contain B220⁺CD43⁺ progenitor cells that express germline μ and IL-7 receptor transcripts. Various non-lymphoid tissues that express *EBF* are apparently normal in homozygous mutant mice, including olfactory neurons in which *EBF* was identified as *Olf-1* (refs 5, 6). Together, these data suggest that *EBF* plays a specific and important role in the transcriptional control of B-cell differentiation at a stage before *Ig* (immunoglobulin) gene rearrangement but after commitment of cells to the B-lymphoid lineage.

Expression of the *EBF* gene is restricted, within the haematopoietic lineage, to cells representing the early stages of B lymphocyte differentiation^{1,4}. *EBF* recognizes a functionally important sequence in the promoter of the *mb-1* gene and regulates its transcriptional activity^{1,2,4}. The *mb-1* gene encodes Ig- α , which is associated with the membrane-bound immunoglobulin receptor and participates in immunoglobulin receptor-mediated signal transduction^{7–10}. *EBF* binds to DNA as a homodimer and contains a DNA-binding domain encompassing a novel type of zinc-coordination motif^{4,5,11}. To determine the role of *EBF* in mouse development, we generated mice that carry a targeted mutation in the *EBF* gene residing on chromosome 11 (ref. 12). In the mutant *EBF* allele, genomic sequences encoding a functionally important portion of the DNA-binding domain of *EBF* were replaced with a *neomycin-resistance* gene cassette (Fig. 1a). Genotypes of mice derived from crossing heterozygous mutant mice were determined by DNA blot analysis (Fig. 1b). Homozygous mutant mice were significantly smaller than their littermates and approximately 30% of them died before 4 weeks of age for unknown reasons.

To examine the effects of the *EBF* mutation on lymphoid development, we analysed peripheral lymphocytes from wild-type and *EBF*^{−/−} mice by flow cytometry with antibodies directed against various lymphoid surface molecules (Fig. 2a–c). Mature B cells, defined by the presence of both B220 and IgM on their cell surface, were detected at high abundance in blood and spleen of wild-type mice but were completely absent in *EBF*^{−/−} mice (Fig. 2a, b). Moreover, no serum IgM was detected in *EBF*-deficient mice (results not shown). This B-cell deficiency was observed in over 70 homozygous mutant mice

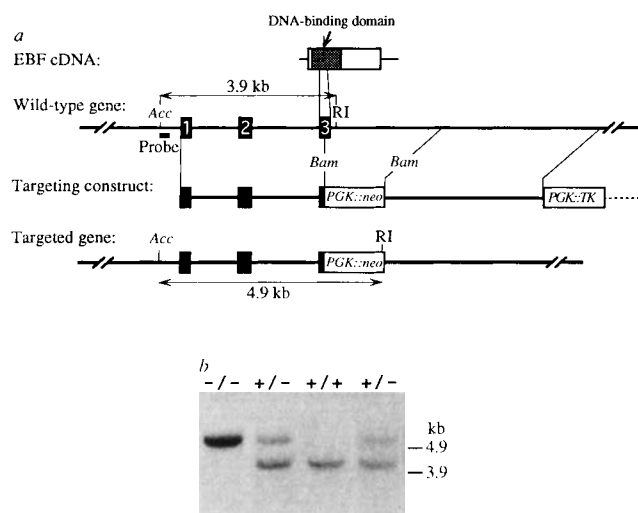


FIG. 1 Inactivation of the *EBF* gene by homologous recombination. **a**, Structure of the *EBF* complementary DNA and partial maps of the

genomic *EBF* locus, the targeting construct and the targeted allele. The first three exons of the wild-type *EBF* gene are shown as solid bars and numbered. Boundaries of homology between the wild-type gene and the targeting construct are denoted by thin lines. The DNA probe and restriction enzymes used in the DNA blot analysis of the wild-type and mutant alleles, as well as the predicted sizes of restriction fragments, are indicated. **b**, Representative DNA blot analysis of mice derived from intercrossing *EBF*^{+/-} mice. Genomic DNA isolated from wild-type, *EBF*^{+/-} and *EBF*^{-/-} mice were digested with *AccI* and *EcoRI* and hybridized with a genomic flanking probe shown above. **METHODS.** Murine genomic *EBF* clones were isolated from a 129/Sv strain genomic library. An 11.2-kb genomic DNA containing 5' exons of the *EBF* gene was subcloned into pBluescript (Stratagene) to generate the gene targeting construct in which a 3.4-kb *Bam*HI fragment was replaced with the 1.4-kb *PGK::neo* gene cassette. The orientation of the *neo* gene is opposite to that of the *EBF* gene. The *PGK::TK* gene cassette was subsequently inserted into a blunt-ended *Sall* site. The gene targeting construct was linearized and introduced by electroporation into D3 embryonic stem cells; clones were doubly selected with neomycin and FIAU for the presence of the *neo* gene and loss of the *TK* gene as described²⁹. 960 embryonic stem (ES) cell clones were screened and three clones that carried the mutation in the *EBF* gene were identified by DNA blot analysis. These heterozygous mutant ES cell clones were used to generate chimaeric mice by microinjection into C57BL/6 blastocysts and *EBF*^{-/-} mice were obtained by crossing *EBF*^{+/-} animals.

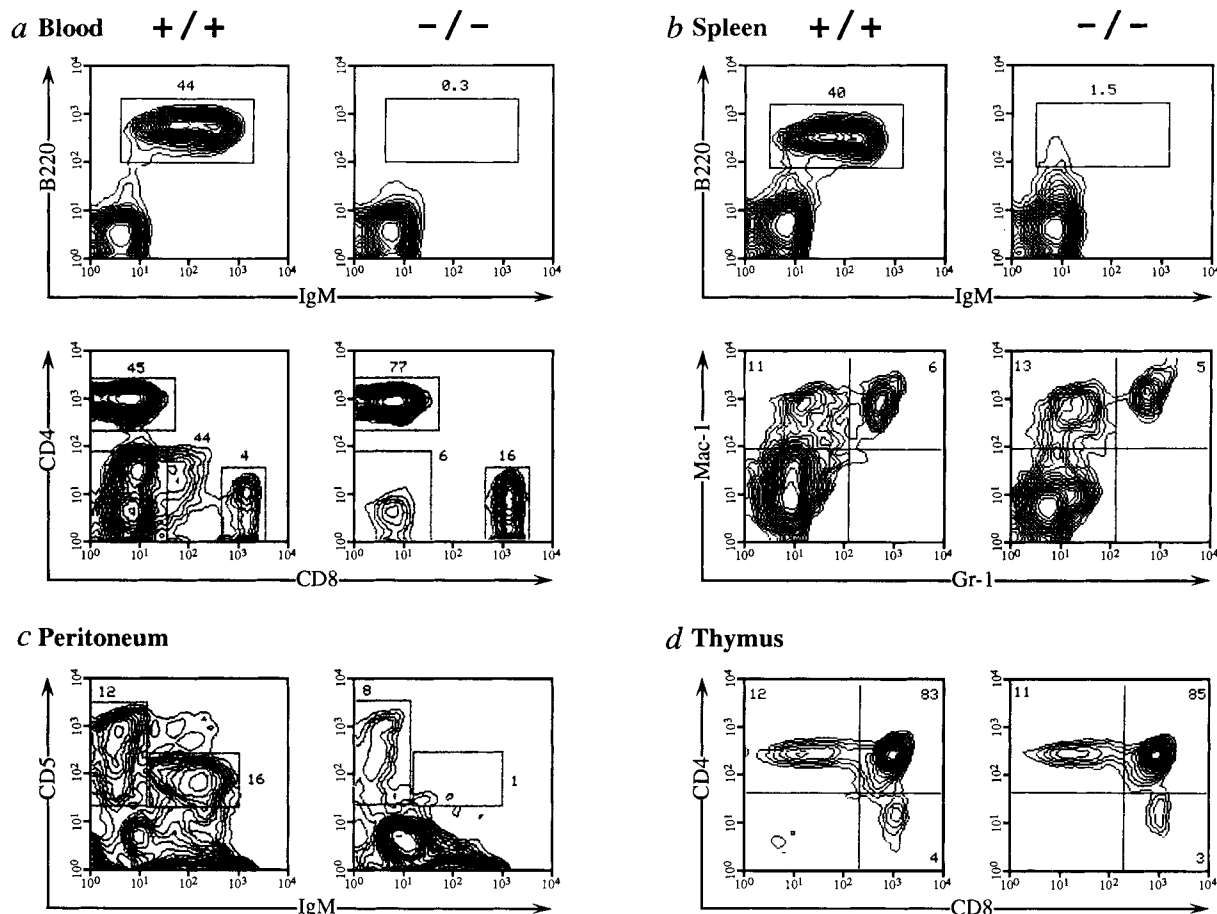


FIG. 2 *EBF*-deficient mice lack mature B cells. **a**, Analysis of peripheral blood lymphocytes by flow cytometry. B cells from the blood of wild-type and *EBF*^{-/-} mice were stained with phycoerythrin (PE)-anti-B220 and fluorescein (FITC)-anti-IgM antibodies and identified by flow cytometry. T cells were stained with PE-anti-CD4 and FITC-anti-CD8. **b**, Splenic B cells were analysed PE-anti-B220 and FITC-anti-IgM and myeloid cells were revealed by PE-anti-Mac-1 and FITC-anti-Gr-1. **c**, Cells from the peritoneal cavity were analysed with PE-anti-CD5 and FITC-anti-IgM. **d**, Thymocytes were stained with PE-anti-CD4 and FITC-anti-CD8. Percentages of cells for the indicated subpopulations are given.

METHODS. Single-cell suspensions of lymphoid tissues were prepared from 8-week-old *EBF*^{-/-} mice and wild-type littermates. Red cells in blood and spleen samples were lysed with 150 mM NH_4Cl , 10 mM KHCO_3 and 0.1 mM EDTA. 10^6 cells were incubated with PE- and FITC-conjugated monoclonal antibodies in 100 μl staining solution (PBS containing 0.3% bovine serum albumin), washed and then analysed using a FACScan (Beckton Dickinson). Dead cells were gated out, based on propidium iodide ($0.5 \mu\text{g ml}^{-1}$) uptake. Data are presented as 5% probability contour plots. Monoclonal antibodies: PE-anti-B220, FITC-anti-CD8, PE-anti-CD4, PE-anti-Mac-1, and FITC-anti-Gr-1 (Caltag), FITC-anti-IgM and PE-anti-CD5 (PharMingen).

analysed, indicating complete penetrance of the phenotype. In addition to the defect in conventional B cells, EBF-deficient mice also lacked both $CD5^+IgM^+$ and $CD5^-IgM^+$ B cells¹³ in the peritoneal cavity (Fig. 2c). In contrast, thymic and peripheral T cells expressing CD4 and/or CD8, and myeloid cells displaying the surface markers Mac-1 and Gr-1, were present in normal numbers in $EBF^{-/-}$ mice (Fig. 2a, b, d).

Differentiation of B lymphocytes occurs in multiple stages¹⁴. In addition to mature B cells, which express IgM on the cell surface, and plasma cells, which secrete antibodies, IgM⁻ precursor B cells can be divided into four groups based on differen-

tial expression of CD43(S7), HSA and BP-1 (refs 15, 16). Cells of fraction A, containing the *Ig heavy chain (H)* locus in its germline configuration, are defined by the surface expression of B220 and CD43 and the absence of HSA and BP-1. Fraction B cells, characterized by a $B220^+CD43^+HSA^+BP-1^-$ surface phenotype, together with fraction C cells expressing all four surface antigens, represent subsequent stages of differentiation that involve rearrangements at the *IgH* locus. Finally, pre-B cells, which express cytoplasmic Ig- μ chain but lack CD43 on the cell surface, undergo *Ig light chain* rearrangement. To identify the stage at which B-cell differentiation is blocked in $EBF^{-/-}$ mice, we used flow cytometry to analyse cells from the bone marrow, which is the primary organ for B lymphopoiesis in adult mice. Similarly to the observation made on peripheral lymphocytes, the $B220^+IgM^+$ mature B-cell population was absent in the bone marrow of EBF-deficient mice. Moreover, the number of $B220^+IgM^-$ cells was severely reduced (Fig. 3a). The bone marrow of $EBF^{-/-}$ mice also lacked the $B220^+CD43^-$ cell populations, whereas $B220^+CD43^+$ cells were present (Fig. 3b). To define the $B220^+CD43^+$ cell population further, we examined these cells for the surface expression of HSA and BP-1. All three (A, B and C) fractions of bone marrow cells were readily detected in wild-type mice, whereas bone marrow cells of EBF-deficient mice lacked both HSA and BP-1 on the cell surface, indicating that only fraction A cells are present (Fig. 3c).

Analysis of bone marrow cells of heterozygous mutant mice revealed an approximately 50% decrease in the numbers of fraction B and fraction C cells relative to wild-type mice, whereas the number of fraction A cells was normal. Moreover, we observed a 10–30% reduction in the number of B cells in the spleen of $EBF^{+/-}$ mice (results not shown). The modest decrease in numbers of B cells in heterozygous mutant mice is probably due to a gene dosage effect rather than to a dominant-negative effect of a truncated polypeptide expressed from the mutant allele, because similar levels of DNA binding by EBF were detected in nuclear extracts from the bone marrow of heterozygous and wild-type mice (results not shown).

To characterize the B-cell deficiency in $EBF^{-/-}$ mice at the molecular level, we examined both the rearrangement status of the *Ig* loci and the RNA expression from various B-cell-specific genes (Fig. 4). We isolated $B220^+$ cells from the bone marrow of wild-type and EBF-deficient mice and prepared genomic DNA and total RNA. Genomic polymerase chain reaction (PCR) analysis with primers designed to detect *Ig* gene rearrangements indicated that D-J_H, V-DJ_H and V-J_κ rearrangements were virtually undetectable in the DNA samples from homozygous mutant mice. As a control, equivalent amounts of amplification products from the *α-actin* gene were obtained from both the wild-type and homozygous mutant DNA samples (Fig. 4a). Analysis of RNA from $B220^+$ bone marrow cells by PCR after reverse transcription (RT-PCR) indicated that EBF-deficient mice lacked transcripts from the *mb-1*, *B29*, *V_{preB}*, *λ5*, *RAG-1* and *RAG-2* genes, and the D-J_H-rearranged *μ* gene (Fig. 4b and results not shown). These genes are normally expressed in fraction B cells and cells representing subsequent stages of early B-cell differentiation^{17,18}. Cells of fraction A are defined only by the presence of the surface markers B220 and CD43 and are likely to include non-B cells that express these surface markers. However, normal levels of expression of the *IL-7 receptor (IL-7R)* gene and the germline *μ* locus, and somewhat reduced expression of the *terminal deoxynucleotidyl transferase (TdT)* gene were detected in $EBF^{-/-}$ mice, suggesting that these mice contain cells committed to the B lymphoid lineage (Fig. 4b). Moreover, transcripts from the mutant *EBF* allele can be detected in these B lymphocyte progenitors, indicating that *EBF* is expressed before the block of differentiation (results not shown). Together with the analysis of surface antigens, these data suggest that B-cell differentiation in EBF-deficient mice is blocked at the transition from fraction A cells to fraction B cells, preventing the production of pro-B cells that undergo D-J_H

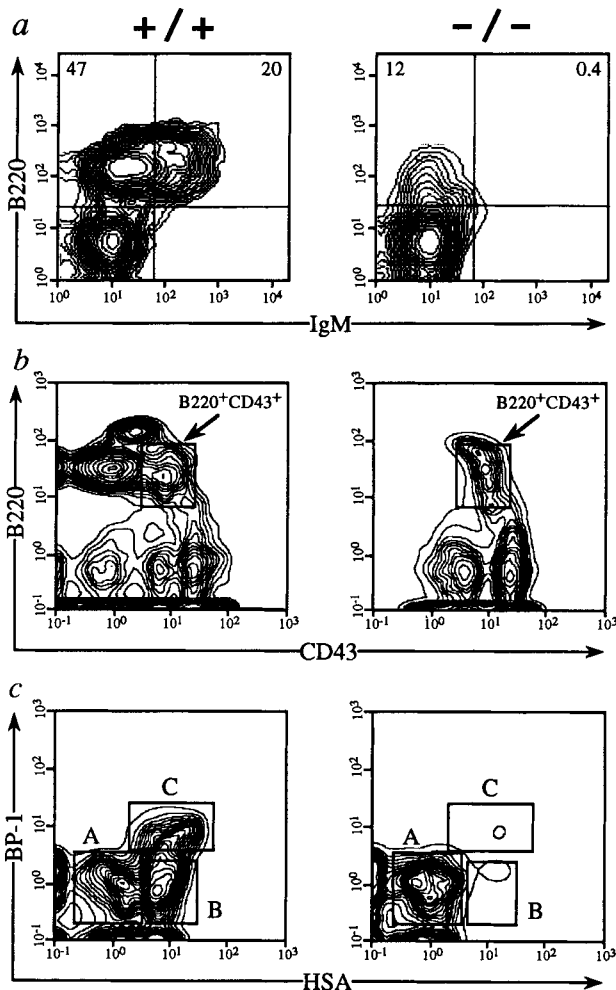
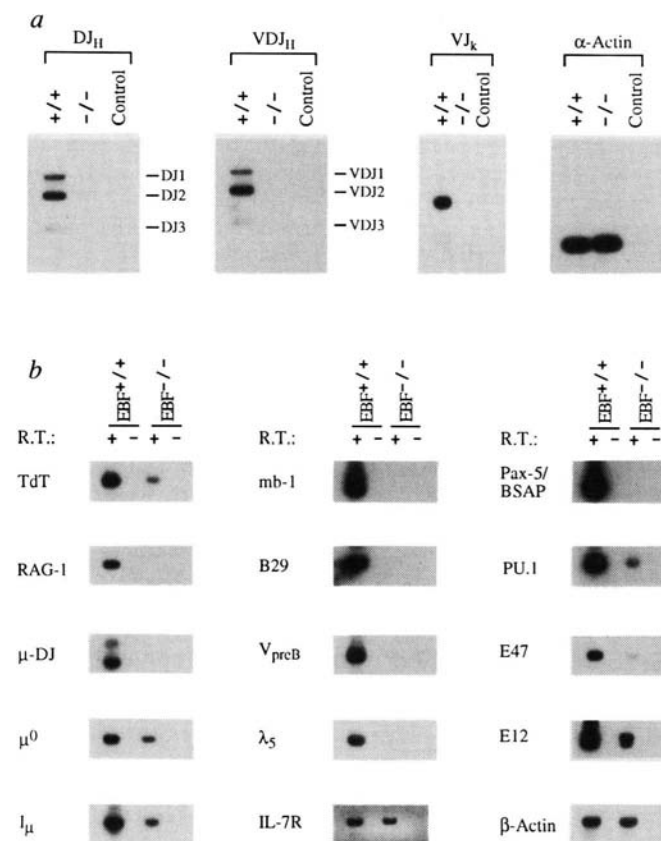


FIG. 3 B-lymphocyte differentiation in EBF-deficient mice is blocked at an early stage. a, Surface expression of B220 and IgM on bone marrow cells of wild-type and $EBF^{-/-}$ mice. Percentages of cells in subpopulations are indicated. b, c, Four-colour FACS analysis of bone marrow cells. The cell surface expression of B220 and CD43(S7) is shown in b. $B220^+CD43^+$ cells were further analysed for the expression of BP-1 and HSA, and plots are shown in c. Fractions A, B and C of pro-B cells are indicated. Fraction A cells represent 0.5% of total bone marrow cells in wild-type mice and 0.6% in $EBF^{-/-}$ mice.

METHODS. Bone marrow cells obtained from 8-week-old mice were stained with PE-anti-B220 and FITC-anti-IgM and analysed by flow cytometry as described in Fig. 2. For four-colour FACS analysis, bone marrow cells were simultaneously stained with allophycocyanin (APC)-anti-B220, FITC-anti-CD43(S7), PE-anti-HSA(M1/69), and biotinylated anti-BP-1(6C3) (PharMingen) on ice for 15 min, washed 3 times with staining buffer (PBS/0.3% BSA), then incubated with Texas Red (TR)-streptavidin for an additional 15 min, and finally washed 3 times with staining buffer. Stained cells were analysed with a modified, dual-laser FACS II and FACS-DESK software (Stanford University). Data are shown as 5% probability contour plots. Dead cells were gated out, based on forward and side scatter.



rearrangement. Thus B-cell differentiation in these mutant mice is arrested at an earlier stage than in the RAG-1 or RAG-2-deficient mice, which contain progenitor B cells of fractions A, B and C (refs 19–22).

To gain some insight into the genetic hierarchy of transcriptional regulators of B lymphopoiesis, we examined RNA from B220⁺ bone marrow cells for the presence of transcripts from the *E2A* and *Pax-5* genes, which have been recently shown to regulate B lymphocyte differentiation^{23,25}. No *Pax-5* transcripts that encode BSAP were detected in the *EBF*^{-/-} mice, suggesting that EBF is required at an earlier stage of B lymphopoiesis than BSAP (Fig. 4b). In contrast, transcripts from the *E2A* gene, encoding E47 and E12 polypeptides, were detected in B220⁺ bone marrow cells of *EBF*-deficient mice, albeit at a significantly lower level. Likewise, the level of transcripts of the *PU.1* gene, which is required for myeloid and lymphoid development²⁶, was reduced in *EBF*^{-/-} mice (Fig. 4b). This reduction in the numbers of E47, E12 and *PU.1* transcripts is probably due to a lower level of expression of these genes in fraction A cells than in the total B220⁺ bone marrow cells. However, we cannot rule out that the mutation in the *EBF* gene may affect the expression of these regulatory genes in B lymphocytes. *E2A*^{-/-} mice lack B cells that undergo D–J_H rearrangement and possibly fail to produce the progenitor cells representing the initial stage of the B-cell lineage^{23,24}. In *EBF*^{-/-} mice, these progenitor cells are present in normal numbers (Fig. 3), suggesting that EBF is not required for the determination of the B-cell lineage. Both *EBF* and *E2A* genes, however, may act downstream of the transcription factor genes *PU.1* and *Ikaros*, which have been shown to control events preceding the commitment of cells to the B lymphoid lineage^{26,27}.

Although the *mb-1* gene is the only known target for EBF in B lymphopoiesis so far, it is likely that EBF may also regulate

FIG. 4 Immunoglobulin gene rearrangement and gene expression in B220⁺ bone marrow cells. *a*, *IgH* and *Igk* gene rearrangements. PCR reactions were done with genomic DNA isolated from the B220⁺ bone marrow cells of wild-type or *EBF*^{-/-} mice with primers specific for D–J_H, V–DJ_H and V–J_k rearrangements^{18,30}. PCR reactions without DNA templates served as controls. On long exposure of the autoradiogram, very weak signals for D–J_H rearrangement were detected in the DNA sample of *EBF*^{-/-} mice, consistent with the low frequency of this type of rearrangement in fraction A pro-B cells¹⁷. *b*, Analysis of gene expression in B220⁺ bone marrow cells. Reactions without the addition of the reverse transcriptase (R.T.) (denoted by –) were used to control for contamination of the RNA samples by genomic DNA.

METHODS. Bone marrow cells from 12-week-old mice were incubated with biotinylated anti-B220 (Caltag), washed, and then incubated with Dynabeads M-280 streptavidin (Dyna) with gentle shaking at 4 °C for 30 min to purify B220⁺ cells according to the manufacturer's instruction. Genomic PCR was done with DNA isolated from B220⁺ bone marrow cells, under the following amplification conditions: 45 s at 95 °C, 45 s at 55 °C and 1.5 min at 72 °C, for 30 cycles. For analysis of RNA transcripts, total RNA from B220⁺ bone marrow cells was isolated by the guanidinium method and first-strand cDNA was synthesized with M-MLV reverse transcriptase (Gibco-BRL) as described¹⁷. The amounts of cDNA were normalized to the RT–PCR product of β-actin. Amplification conditions were as follows: 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, for 30 cycles. PCR products were separated by gel electrophoresis, blotted onto Hybond N⁺ membrane (Amersham) and hybridized with specific DNA probes. The PCR product for IL-7R was revealed by ethidium bromide staining and recorded with a digital imaging system (Alpha Innotech Corp.). The PCR primers of D_H, V_H, J_H3, V_k, J_k, α-actin, β-actin, mb-1, TdT, RAG-1, μ-DJ, μ⁰, I_μ, V_{preB} and E47 were described previously^{17,18,24,30}. The other PCR primers were as follows: B29, 5'-GGTGAGCCGGTACCAGCAATG and 5'-AGTTCGGTGCCACAGCTGTGCG; λ₅, 5'-TGTTGAAGTTCTCCTCTGCTG and 5'-ACCACCAAAGTACCTGGGTAG; IL-7R, 5'-AGCTGTTTCTGGAGAAAGTGG and 5'-AACGACTTTCAGGTCA-GAGGG; Pax-5/BSAP, 5'-CTACAGGCTCCGTGACGCAG and 5'-GTCTCGGCCTGTGAATAGG; PU.1, 5'-CGGATGACTTGGTTACTTACG and 5'-GTAGGAAACCTGGTGACTGAG; E12, 5'-GACGCCGAAGAGGACAAGAA and 5'-TGTTGCAGGATGAGCAGTTT.

other genes involved in B-cell differentiation. In addition to the expression of *EBF* in B cells, transcripts from this gene can be detected in adipocytes⁴, in multiple regions of the developing brain, and in olfactory neurons (M. Arnaud and R.G., unpublished observations), in which the same gene was independently cloned as *Olf-1* (ref. 5). Histological examination of the brain and the olfactory epithelium in *EBF*^{-/-} mice, however, did not reveal any apparent abnormalities. Moreover, preliminary experiments indicated that transcripts of the *OMP* and *G_{olf}* genes, which have been implicated as targets for Olf-1 in the olfactory neurons^{5,6}, and transcripts of the adipocyte-specific *aP2* gene²⁸ can be detected in *EBF*-deficient mice (H.L., I. Farinas and R.G., unpublished observations). Although EBF may be functionally redundant with a closely related factor in non-lymphoid tissues, it clearly plays a specific and essential regulatory role for the differentiation of cells of the B lymphoid lineage. □

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Specific and redundant roles of Src and Fyn in organizing the cytoskeleton

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MOUSE embryos lacking Csk, a negative regulator of Src family kinases, exhibit defects in neurulation and die at mid-gestation^{1,2}. To determine the role of activated Src family kinases in the *csk*⁻ phenotype, we have introduced mutations in the *src* and *fyn* genes^{3,4} into the *csk*⁻ mutant background. Genetic analysis reveals that *src*, but not *fyn*, is partly epistatic to the *csk* gene. Biochemical analysis indicates that several cytoskeletal proteins are hyperphosphorylated on tyrosine residues in *csk*⁻ cells. Regulation of cortactin and tensin hyperphosphorylation is Src-dependent, whereas focal adhesion kinase and paxillin hyperphosphorylation is partly dependent on both Src and Fyn. Furthermore, the *src*⁻ mutation can restore the normal distribution of cortactin and partly correct filamentous actin organization in *csk*⁻ cells. Thus, Src family kinases have both specific and overlapping functions in regulation of the cytoskeleton. The disturbance of these functions may be a molecular basis for the phenotype exhibited by *csk*⁻ mutants.

Loss-of-function mutations in the *src* gene family do not affect mouse embryogenesis despite the prediction that Src family tyrosine kinases (PTKs) play a role in growth and differentiation^{5–7}. Although this result may be due to functional overlap among the family members, analysis of compound mutations is only corroborative; it does not prove this hypothesis⁸. Here we have analysed the relative contributions of different Src family kinases to the *csk*⁻ phenotype in an attempt to identify unique and shared functions.

Csk is a cytoplasmic PTK that has two amino-terminal protein-protein interaction domains (Src homology 2 and 3), and a carboxy-terminal catalytic domain^{9–11}. *In vivo* studies indicated that Csk is required for mouse development^{1,2}. Embryos lacking Csk die at around embryonic day 9 to 10 (E9–10), and exhibit a complex phenotype including defects in the neural tube and

notochord, an inability to continue the turning process, and a failure of the allantois to connect with the chorion, preventing the formation of the umbilical cord and placenta. Cells isolated from *csk*⁻ embryos have decreased phosphorylation of the negative regulatory site of Src (Tyr 527) and increased specific activity of at least three Src family PTKs (Src, Fyn and Lyn).

To help understand how abnormally activated Src family kinases can lead to the *csk*⁻ phenotype, *src* or *fyn* mutant mice were crossed with the *csk*⁻ mice. A mutation in *src*, but not *fyn*, suppressed the *csk*⁻ phenotype (Fig. 1). Two defects in *csk*⁻ embryos, the inability to turn about the anteroposterior axis and to complete the connection of the allantois to the chorion, were restored in *src*⁻*csk*⁻ embryos (Fig. 1). In these embryos, the notochord and neural tube defects also were not as pronounced as in *csk*⁻ embryos (not shown). The *fyn*⁻*csk*⁻ mutant embryos were indistinguishable from *csk*⁻ embryos (Fig. 1b–d). Although

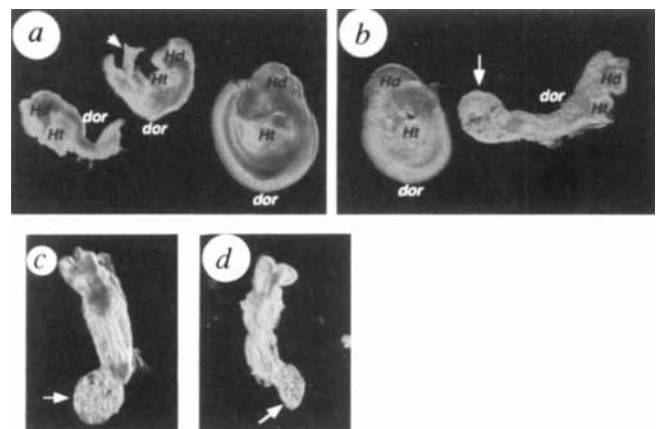


FIG. 1 Partial epistasis of *src* to *csk*. **a**, Lateral views of *src csk* double homozygous (centre), *csk* single homozygous (left), and normal (right) embryos at embryonic day (E) 10.0. The arrowhead indicates the allantois that had been connected to the chorion in the double mutant **b**, Dorsolateral views of *fyn csk* double-mutant (right) and normal (left) embryos at E9.5. **c**, **d**, Dorsal views of a *fyn csk* embryo (**c**) and a *csk* embryo (**d**) at E9.5. *src csk* double-mutant embryos were able to rotate their body about the anteroposterior axis and to connect their allantois to the chorion, unlike *csk* single or *fyn csk* double mutants. Note that all embryos mutant for *csk* failed to close their cephalic folds, and that *csk*⁻ and *fyn csk*⁻ embryos had an enlarged and hydroptic allantois (arrows), which failed to connect to the chorion (**b–d**). The dorsoventral axis in **a** and **b** is shown by indicating the dorsal side (dor). Ht, heart; Hd, head structure.

METHODS. Individual loss-of-function mutations were created by homologous recombination in embryonic stem cells^{1,3,4}. A total of 161 embryos were collected between E9.5 and E10.5 from *src*^{+/-}*csk*^{+/-} parents, of which 9 embryos were *src*^{-/-}*csk*^{+/-} double-homozygous mutants consistent with Mendelian expectations of 1/16. A total of 32 embryos were collected between E9.5 and E10.5 from *fyn*^{+/-}*csk*^{+/-} parents, of which 2 embryos were *fyn*^{-/-}*csk*^{+/-} double homozygous mutants. An additional 20 embryos were collected at E9.5 from *fyn*^{+/-}*csk*^{+/-} parents, of which 3 embryos were *fyn*^{-/-}*csk*^{+/-} double-homozygous mutants. Thus, a total of 5 *fyn*^{-/-}*csk*^{+/-} double-homozygous mutants were collected. Hybrid (129Sv:C57BL/6) animals were used for these crosses. The polymerase chain reaction (PCR) was used to genotype embryos for the *src* and the *csk* loci¹. For the *src* locus, a forward primer downstream of the initiator ATG codon in exon 2 (5'-AGCAAC-AAGAGCAAGCCCAAGGAC-3') and a reverse primer approximately 30 bp downstream of the second SacI site in exon 2 (5'-GTGACGGTGTCCGAGGAGTTGAAG-3') were used to amplify a 240-bp fragment from the wild-type allele. The forward primer and a neo reverse primer (5'-TCATAGCCGAATAGCCTCTCCAC-3') amplify a 350-bp fragment from the *src* mutant allele. PCR genotyping for the *csk* locus was performed as described¹. The genotype was determined by the presence or absence of the wild-type or mutant fragment in an ethidium-bromide-containing agarose gel after electrophoresis. Genotyping for the *fyn* locus was performed by Southern analysis as described⁴.

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the rescue of some aspects of the *csk*⁻ phenotype by the *src*⁻ mutation is significant, the double-mutant embryos still died around E10–11 with unclosed cephalic folds. This epistasis indicates that Src is a downstream mediator of the *csk*⁻ phenotype; however, other parallel effectors must exist because the rescue was only partial.

To explore the underlying molecular interactions implied by the genetic study, we have conducted biochemical analyses on the potential targets of Src-family kinases (Figs 2 and 3). Transformation by *v-src* causes a number of phenotypic changes such as reduced adherence and altered cell morphology due to disrupted cytoskeletal structures and changes in the extracellular matrix^{12,13}. Correlating with these changes is the hyperphosphorylation of proteins that have been proposed to regulate the cytoskeletal architecture and cell-cell interactions. These include: (i) proteins involved in integrin-mediated signalling such as paxillin¹⁴, focal adhesion kinase (Fak)¹⁵ and tensin¹⁶; (ii) proteins involved in cadherin-mediated cell-cell interactions such as p120 (ref. 17) and β -catenin^{18,19}; and (iii) other cytoskeletal proteins such as cortactin²⁰ and actin-filament-associated protein (AFAP)²¹ (see ref. 13 for review). In our study, tyrosine phosphorylation of cortactin, tensin, p120, Fak and paxillin was increased in *csk*⁻ cells (Fig. 2b). We did not detect

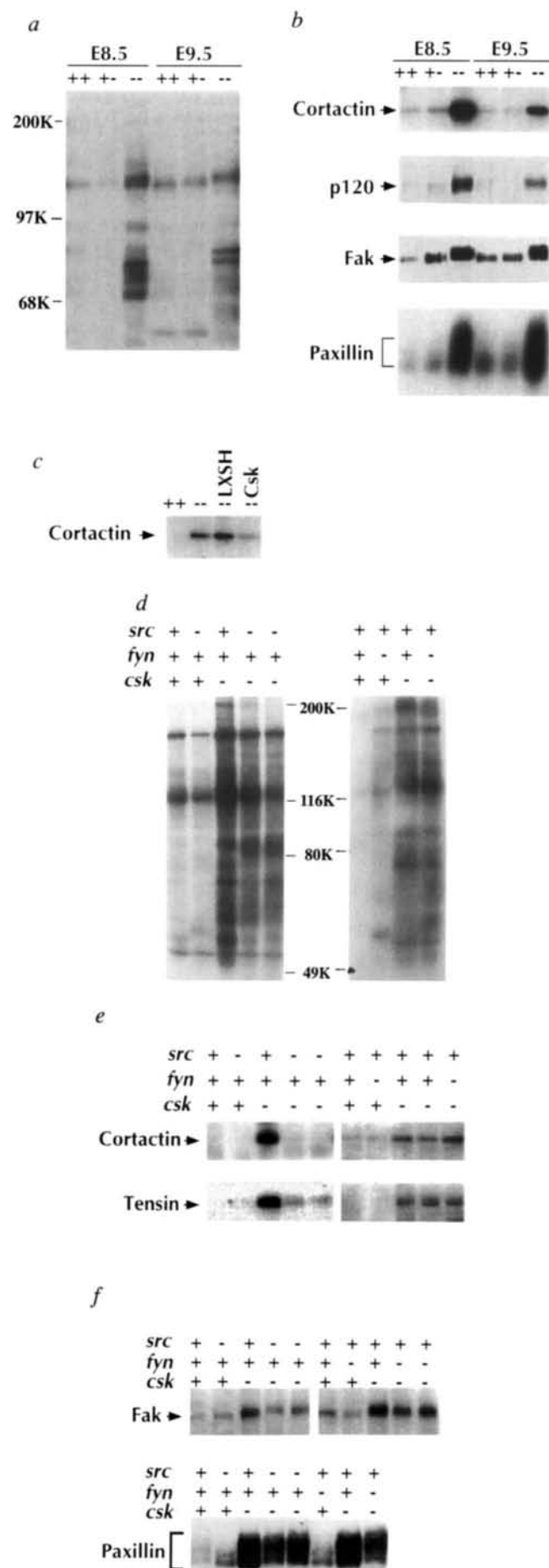


FIG. 2 Increased tyrosine phosphorylation on *v-Src* substrates in *csk* cells. **a**, An anti-phosphotyrosine immunoblot analysis of total cell lysates isolated from embryonic fibroblasts. Relative molecular mass is indicated at the left. **b**, Tyrosine phosphorylation on *v-Src* substrates in *csk*⁻ cells. **c**, The increased phosphorylation on cortactin in *csk*⁻ cells is due to loss of Csk. ++, wild-type; +, heterozygous; -, homozygous for the *csk*⁻ allele. LXSH, *csk*⁻ cells infected with a retrovirus vector. Csk, *csk*⁻ cells infected with a virus transducing a *csk* complementary DNA. Cortactin phosphorylation was increased 15-fold and 20-fold in *csk*⁻ cells and LXSH control cells, respectively, compared with wild-type cells. In *csk*⁻ cells that have a *csk* transgene, the increased phosphorylation was decreased 80% compared with the LXSH vector control cells. The residual phosphorylation in the *csk*⁻ transgenic rescue cells may be due to partial inactivation of the *csk* transgene, because it was difficult to maintain high levels of *csk* expression (results not shown). **d**, An anti-phosphotyrosine immunoblot analysis of total cell lysates isolated from double mutant cell lines. **e**, Src-dependent tyrosine phosphorylation on cortactin and tensin. The genotypes of the cell lines are indicated on top of each blot. +, wildtype or heterozygous; -, homozygous for the corresponding mutant allele. **f**, The elevated tyrosine phosphorylation on Fak and paxillin is due to more than one member of the Src family kinases. Introducing either the *src*⁻ or *fyn*⁻ mutation into the *csk*⁻ background reduced the elevation of Fak and paxillin phosphorylation by 30% or 20% compared with *csk*⁻ cells, respectively. Note that the basal levels of Fak and paxillin phosphorylation in wild-type cells are approximately 30–40% of those in *csk*⁻ cells. Anti-phosphotyrosine immunoblots after immunoprecipitation for *v-Src* substrates are shown in **b**, **c**, **e**, **f**. The amount of each immunoprecipitated protein differed little between the cell lines (not shown). Data in **c–f** are from cells isolated from E9.5 embryos. Similar data were obtained with two sets of E9.5 cells and two sets of E8.5 cells. Each set of cell lines shown in individual panels are isolated from littermate embryos.

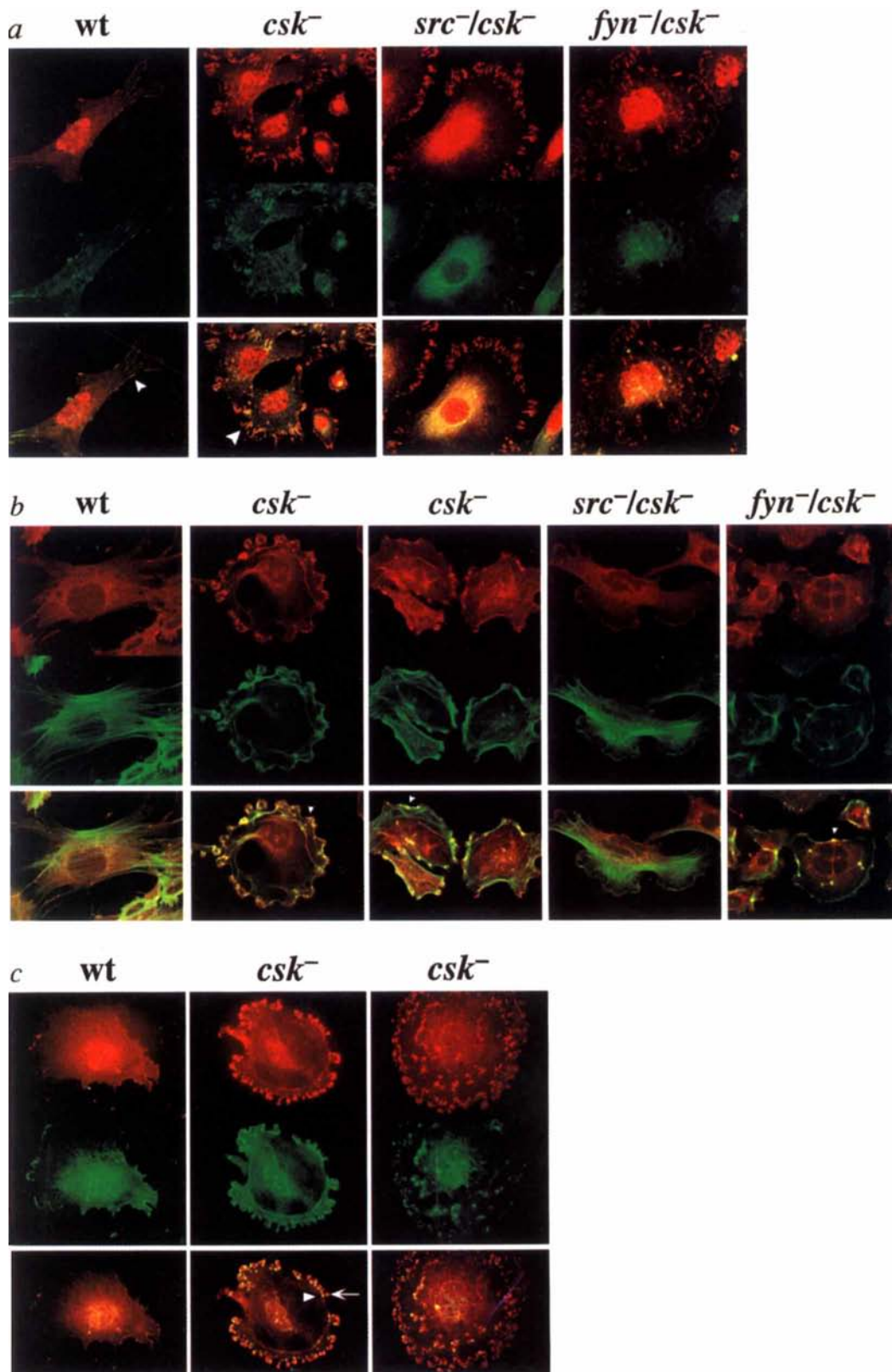
METHODS. Cells isolated from embryos were immortalized with simian virus 40 large T antigen as described¹. Monoclonal antibodies for cortactin, Fak, p120, tensin and paxillin (Zymed) were used to immunoprecipitate these proteins from total cell lysates (500 µg protein) prepared in RIPA buffer. Immunoprecipitated proteins were separated by electrophoresis in an SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane in Tris-glycine buffer containing methanol and SDS. The presence of phosphotyrosine residues in the protein was determined by probing the membrane with a monoclonal anti-phosphotyrosine antibody (4G10; UBI) and visualized by enhanced chemiluminescence (ECL; Amersham) using a secondary anti-mouse antibody conjugated with peroxidase. The control cell line in **c** was a *csk*⁻ embryonic fibroblast line that expresses a *csk* transgene³⁰. All reactions were done at room temperature. Levels of tyrosine phosphorylation on immunoblots were measured by densitometric tracing of the X-ray film (Molecular Dynamics).

increased tyrosine phosphorylation on AFAP or β -catenin (results not shown). These results are consistent in part with those of Nada *et al.*, who found greater tyrosine phosphorylation on cortactin and paxillin in a *csk*⁻*p53*⁻ cell line²². Moreover, in *csk*⁻ embryo extracts, cortactin was found to be hyper-

phosphorylated²², suggesting that the increase in phosphorylation observed in our cell lines also faithfully reflects changes in the embryos. In contrast to our results, however, Nada *et al.* did not observe hyperphosphorylation of p120 and Fak; this discrepancy might be due to the difference in the derivation of

FIG. 3 Effects of loss of Csk on focal adhesion structures and the cytoskeleton. **a**, Immunofluorescence staining with phosphotyrosine (red) and paxillin (green) antibodies to show focal adhesion structures in mutant cell lines. The lower frame in each panel is a superimposed image of phosphotyrosine and paxillin staining. Yellow-orange indicates an overlap of phosphotyrosine and paxillin staining. The white arrowheads indicate a focal adhesion structure. **b**, Immunofluorescence staining of cortactin (red) and filamentous (F-) actin (green) in mutant cell lines. The lower frame in each panel is a superimposed image of cortactin and F-actin staining. Yellow indicates an overlap of cortactin and F-actin staining. The white arrowheads indicate a podosome-like structure. **c**, Immunofluorescence staining of cortactin (green) and phosphotyrosine (red) in primary embryonic fibroblasts. The lower frame in each panel is a superimposed image of cortactin and phosphotyrosine staining. Yellow indicates an overlap of cortactin and phosphotyrosine staining (arrowhead). The arrow indicates phosphotyrosine staining that does not overlap with cortactin staining.

METHODS. The cell lines described in Fig. 2 as well as primary fibroblastic cells at passage 2 isolated from E9.5 embryos were used in these experiments. Cells were plated on poly-L-lysine-coated cover slips. After overnight culture, the cells were fixed with paraformaldehyde (40g l⁻¹) in PBS for 5–10 min, permeabilized for 5 min in Triton X-100 (2g l⁻¹) in PBS and blocked for 10 min with BSA (2g l⁻¹)/Triton X-100 (2g l⁻¹) PBS. The cells were incubated with a mouse monoclonal antibody specific for cortactin or paxillin, polyclonal antibodies specific for phosphotyrosine or with fluorescein-conjugated phalloidin (Molecular Probes) for F-actin. Anti-rabbit or anti-mouse antibodies conjugated with Texas Red or fluorescein (Jackson Immuno-research Labs) were used as a secondary antibody for double staining. Immunofluorescence images were collected in a scanner-laser confocal microscope (MRC 600, Bio-Rad). Computer images were coloured with the Adobe Photoshop program. The nuclear phosphotyrosine staining in **a** was specific



for the polyclonal antibodies because the monoclonal anti-phosphotyrosine antibody used in Fig. 2 (4G10) did not stain nuclei (results not shown).

the cell lines, and to the fact that only one *csk*⁻*p53*⁻ cell line was examined.

To determine which Src family kinase was responsible for the increased phosphorylation of each substrate, we have examined cell lines from *src*⁻*csk*⁻ and *fyn*⁻*csk*⁻ embryos. We have observed a decrease in total cell phosphotyrosine in *src*⁻*csk*⁻ but not *fyn*⁻*csk*⁻ cells compared with *csk*⁻ cells (Fig. 2d). The hyperphosphorylation of cortactin and tensin is suppressed in *src*⁻*csk*⁻ cells but not in *fyn*⁻*csk*⁻ cells (Fig. 2e). Although these results could be due to a direct or indirect regulatory mechanism, they suggest that the phosphorylation of these proteins is dependent on Src. Both Fak and paxillin phosphorylation was slightly decreased in *src*⁻*csk*⁻ and *fyn*⁻*csk*⁻ cells, indicating a role for multiple Src family kinases (Fig. 2f). Consistent with this hypothesis, a *yes* mutation⁸ synergistically attenuated tyrosine phosphorylation of Fak and paxillin in a triple-mutant cell line for the *csk*, *src* and *yes* genes (results not shown). However, because the mutated *yes* gene still generates a catalytically inactive protein that contains the amino-terminal half of Yes (not shown), these results could be due to a neomorphic function of the truncated protein. These changes in phosphorylation are not due to differences in the levels of expression of these proteins in the various cell lines (results not shown).

Although the role of tyrosine phosphorylation in the function of these cytoskeletal proteins is not fully understood, studies on *v-src*-transformed cells have shown that these increases in tyrosine phosphorylation correlate with cytoskeletal rearrangements and changes in the subcellular localization of some of the substrates. To determine whether such changes occur in *csk*⁻ cells, we have analysed focal adhesion structures (using antibodies to paxillin) and actin cables (using phalloidin and antibodies to cortactin) (Fig. 3). In *csk*⁻ cells, focal adhesion structures were abnormal and contained tyrosine-phosphorylated proteins (Fig. 3a). There were increased numbers of these structures distributed throughout the inner cell surface proximal to the poly-L-lysine-coated tissue-culture matrix. Although the *src*⁻ and *fyn*⁻ mutations can slightly reduce the greater tyrosine phosphorylation of two focal adhesion proteins, Fak and paxillin, these mutations had little effect on the abnormal focal adhesion structures in *csk*⁻ cells. Because Fak and paxillin phosphorylation is partly dependent on both Src and Fyn (Fig. 2f), it will be of interest to determine the combined effect of *src*⁻*fyn*⁻ mutations on the abnormal focal adhesion structures of *csk*⁻ cells. When cells were immunostained for phosphotyrosine and F-actin, podosome-like structures²³ containing aggregated F-actin were also stained for phosphotyrosine. We detected a number of phosphotyrosine-containing structures that did not overlap with the F-actin staining, presumably representing abnormal focal adhesion structures (results not shown; see also Fig. 3a). Cortactin and F-actin in *csk*⁻ cells were aggregated in podosome-like structures as previously reported in *src*-transformed cells²⁴ (Fig. 3b). Interestingly, the *src*⁻ mutation restored the normal distribution of cortactin and partly corrected the impaired formation of actin stress fibres in *csk*⁻ cells. But the *fyn*⁻ mutation had little effect on these structures (Fig. 3b). Thus, we have shown that co-localization of cortactin and F-actin in podosome-like structures correlates with the Src-dependent phosphorylation of cortactin. Furthermore, the abnormal localization of cortactin we found in these cell lines was also observed in primary *csk*⁻ cells (Fig. 3c). Consistent with the findings on the immortalized cell lines (Fig. 3a, b), there is an overlap between the cortactin and phosphotyrosine staining, although other structures, such as abnormal focal adhesions (see Fig. 3a), were also stained with anti-phosphotyrosine antibodies (Fig. 3c).

Cortactin is also found in the cell-matrix adhesion junctions of normal osteoclasts (bone-resorbing cells) and this localization is lost in *src*⁻ osteoclasts, which are unable to resorb bone matrix²⁵ (B. F. Boyce, T. Yoneda and G. R. Mundy, personal communication). These observations also suggest that Src plays a biological role in the regulation of cytoskeletal structures by

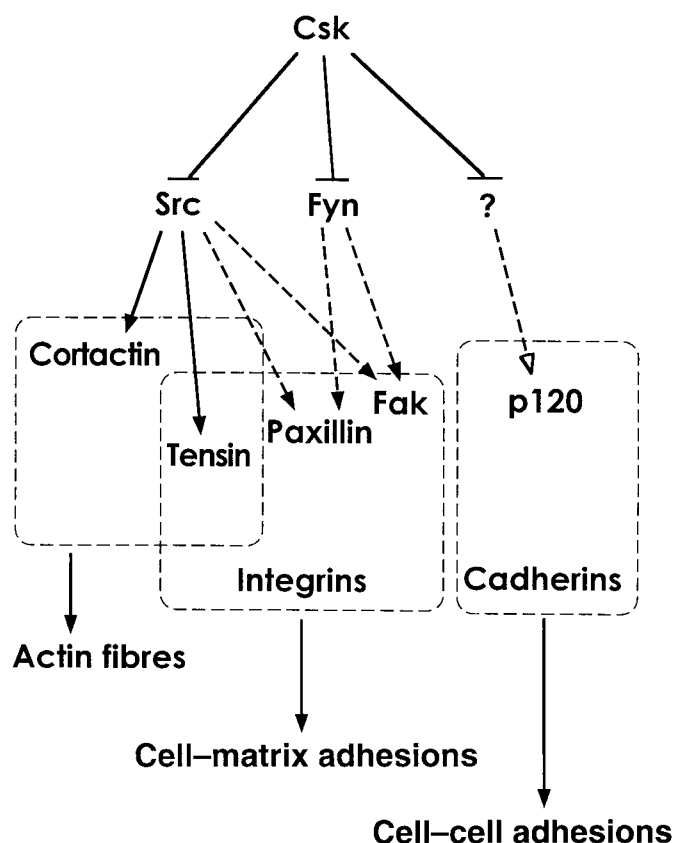


FIG. 4 Molecular interactions regulated by Csk. Csk negatively regulates its effectors directly downstream, including Src and Fyn. These downstream effectors regulate their targets, which were initially identified as cytoskeletal proteins important for cell transformation by *v-src* (ref. 13). Solid lines with closed arrowheads indicate specific regulation of tyrosine phosphorylation. Broken lines with closed arrowheads indicate shared regulation of tyrosine phosphorylation. Broken lines with open arrowheads indicate a hypothetical regulation of tyrosine phosphorylation.

phosphorylating cortactin. None the less, it is possible that other Src-family kinases may play a role in phosphorylating cortactin at a later stage of development or in other cell types. It is not clear whether this regulation is important for the phenotypic rescue observed in *src*⁻*csk*⁻ embryos, but this may be addressed by generating mutations in the *cortactin* gene.

The inability of the *fyn*⁻ mutation to ameliorate the *csk*⁻ phenotype (Fig. 1) or the abnormal cytoskeletal structures (Fig. 3) suggests that at this stage of mouse development the function of Fyn may be unnecessary or redundant (see Fig. 2). Alternatively, there may be a threshold effect; that is, the fivefold increase in Fyn activity due to the loss of Csk^{1,2} could be too small to cause any biological consequences. In addition, Fyn expression during mouse development may be more restricted temporally and spatially than that of Src²⁶. Therefore it is possible that the *fyn*⁻ mutation rescues the *csk*⁻ phenotype in a small subpopulation of cells in the embryo and that we were unable to detect such subtle changes.

Our results suggest that Csk orchestrates the activity of the Src family PTKs and thereby organizes the cytoskeleton through molecules involved in cell adhesion (Fig. 4). Thus, the abnormal interactions of these molecules in *csk*⁻ embryos may perturb proper morphogenetic movements that are required for normal embryogenesis²⁷. Similarities between the *csk*⁻ phenotype and the *fibronectin*²⁸, *α5 integrin*²⁹ and *fak* phenotypes (S. Aizawa, personal communication) also support this hypothesis. Further studies, such as making mutations in the downstream targets of

Src family kinases and other effectors of Csk, will help address more precisely how these molecules are involved in the *csk*⁻ phenotype and in Src family PTK signalling. □

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Three-dimensional structure of a tubulin–motor-protein complex

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THE kinesin superfamily is a class of microtubule-based mechano-enzymes involved in intracellular transport and chromosome movements. Molecules that move towards either the plus end or the minus end of microtubules are represented within the family. The motor domains of these molecules exhibit considerable sequence homology and contain both the ATP- and microtubule-binding sites (reviewed in refs 1, 2). Here we focus on non-claret disjunctional (*ncd*), a minus-end-directed motor involved in chromosome segregation in meiosis and early mitosis in *Drosophila*^{3–6}. We have calculated a three-dimensional map of tubulin sheets decorated with monomeric recombinant *ncd* motor domains⁷ by negative-stain electron microscopy and image analysis. Comparisons with a control structure of tubulin alone reveal that each motor domain binds to the crest of a single protofilament, making extensive contacts with both the α and β tubulin monomers. Binding of the motor domain results in significant conformational changes in both of the tubulin monomers.

The tubulin sheets we studied are the flattened walls of microtubules that form during the early stages of polymerization under certain conditions. They have the appearance of long, narrow, two-dimensional crystals^{8,12} (Fig. 1). Using tilt series reconstruction methods¹³, we calculated three-dimensional maps of tubulin sheets and of sheets decorated with the motor domain of *ncd* at ~20 Å resolution. The data from 123 images of sheets (–60.8 to +62.4° tilt) and 195 images of *ncd*-decorated sheets (–61.6 to +66.4°) were sufficient to determine the continuous variations in amplitude and phase along the lattice lines (see Fig. 2 legend). Regular sampling of curves fitted to the experimental data pro-

vided the Fourier terms used to calculate the three-dimensional maps. Comparisons of the maps and their orthogonal projections provide detailed information on the binding geometry and molecular structure of the *ncd*-tubulin complex.

Structural features in the *xy* projection map calculated from tubulin sheets (Fig. 3a) are similar to those reported by others^{8,10,14,15}. The α and β tubulin monomers are clearly different in negative stain at 20 Å resolution¹⁰, but their identity cannot be assigned so we refer to them as monomer (i) and monomer (ii). Connections between monomers along the protofilament lie at the outer surface of the microtubule (Fig. 3b). Consequently this surface of the protofilament—along which a motor molecule would travel—is remarkably smooth, whereas the inner surface has deep invaginations between the monomers. Looking along the protofilament towards the plus end (Fig. 3c), the projected density distribution suggests that the connections between protofilaments are at low microtubule radius. Assuming that the protofilament sheets flex about this connection, the outer surfaces of adjacent protofilaments would be spaced farther apart in microtubules than is shown in Fig. 3c. This bonding pattern explains the characteristic helically striated inside surface and axially striated outside surface of the microtubule seen by other methods (for example, ref. 16).

In the *xy* projection of the *ncd*-decorated sheet, the characteristic features of both tubulin monomers are obscured by the additional mass (compare Fig. 3a and 3d). The side projection of the decorated protofilament reveals that one motor domain binds to two tubulin monomers (Fig. 3e). Although the main *ncd* peak lies directly above the type (ii) monomer, there is substantial *ncd* density above the type (i) monomer as well. This binding pattern is reminiscent of the situation in actomyosin where a myosin head interacts with two adjacent actin molecules in the filament¹⁷. However, in myosin the minor attachment is in the leading position whereas in *ncd* the smaller connection is in the trailing position. As the mechanism of mechanochemical transduction of the myosins and the kinesins is likely to be different^{18,19}, it is conceivable that directed movement of the *ncd* motor domain could involve sequential formation (or breakage) of the contacts with the two tubulin molecules.

Viewed towards the plus end, the projected density of the decorated protofilament shows that each motor domain binds to the crest of a single protofilament (Fig. 3f). The *ncd*-tubulin complex has a crescent-like appearance in this view: the tubulin monomer appears to be leaning to the left, whereas the *ncd* motor domain leans towards the right.

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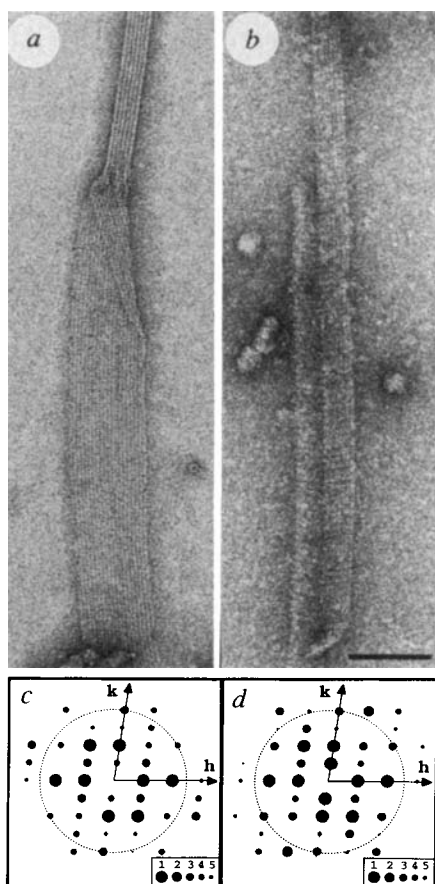


FIG. 1 Images of tubulin sheets (a) and sheets decorated with the motor domain of ncd (b) preserved in negative stain. Representations of the diffraction data (intensity quotient (IQ) plots; see ref. 13) obtained from such images after correction of the lattice for distortions are shown in (c) and (d), respectively. The size of the circle in the IQ plots represents the quality of the diffraction spot. The largest circle (IQ=1) represents a spot with a signal to noise (S:N) ratio of >7, the smaller circles have progressively poorer S:N ratios (for example, IQ 5 represents a S:N ratio of 4). Data with IQs of 1-7 were collected. The dotted circles in c and d are at a resolution of $0.05 \text{ \AA}^{-1}$. Scale bar, $1,000 \text{ \AA}$.

METHODS. The recombinant ncd motor domain was expressed and purified as described⁷. Phosphocellulose-purified tubulin was obtained from bovine brain by published procedures²³ or purchased from Cytoskeleton (Denver, CO). The tubulin from these sources was free of microtubule-associated proteins and other contaminating proteins. Sheets grown from the two preparations were structurally indistinguishable: projection maps were not significantly different. Tubulin at 5 mg ml^{-1} was polymerized for 30 min at 37°C in 80 mM PIPES, pH 6.8, containing 2 mM MgCl_2 , 150 ml per l DMSO, 2 mM GTP, and the resulting microtubules and sheets were stabilized with $20 \text{ \mu M}$ taxol. Dilutions of this solution were adsorbed to fresh carbon films on electron microscope grids and rinsed before negative staining with 2% uranyl acetate. Decoration with ncd was carried out on the grid as follows. After adsorbing the tubulin sheets, the grids were rinsed twice with GTP-free buffer and then incubated for 3 min with a solution containing 3.5 mg ml^{-1} ncd motor domain ($M_r 41.3\text{K}$), 10 mM PIPES, pH 7.2, containing 130 mM NaCl, 2 mM MgCl_2 , 1 mM EGTA, 1 mM DTT, 2 mM AMP-PNP (ref. 24) before negative staining. Grids were examined in a Philips CM12 electron microscope operating at 100 kV . Low-dose images were recorded at a nominal magnification of $\times 45,000$.

A difference map of the ncd motor domain (Fig. 4a) emphasizes the asymmetric nature of the molecule. The shape is consistent with the $3.5 \text{ \AA}$ map calculated by X-ray crystallography (E.P.S., unpublished observations). The overall dimensions are $\sim 50 \times 60 \times 40 \text{ \AA}$ (in x, y, z). The motor is elongated axially and its shape mimics the slight side-to-side variations in position of the underlying flat outer surface of the protofilament. An

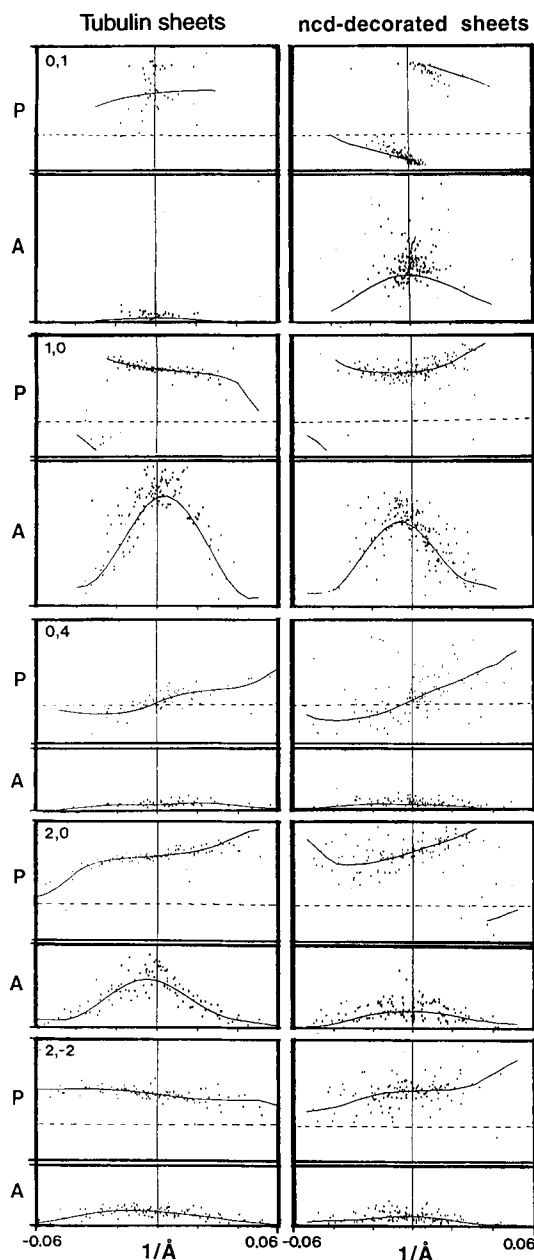


FIG. 2 Representative lattice lines from tubulin sheets (left) and ncd-decorated sheets (right) showing the experimental values (dots) of amplitude (A) and phase (P) obtained from the images, together with the smooth curves fitted to these data by the program LATLINE²⁵. All amplitude plots have a common scale. Complete data sets or contoured map sections are available from the authors.

METHODS. The three-dimensional (3D) analysis was carried out by standard procedures^{13,26}. Briefly, a number of tilt series ($+60^\circ$ to -60° , 7.5° increment) were recorded. Well-ordered areas of the sheets were digitized with a microdensitometer using spot and step sizes equivalent to $5.56 \text{ \AA}$ at the specimen. Image analysis, including unbending¹³, was performed with SPECTRA²⁷. Experimental data from 123 images (tubulin sheets) and 195 images (ncd-decorated sheets) were separately merged and refined to a common phase origin assuming only translational symmetry (P1). Initially, data from sheets in opposite orientations on the grid were separately merged. However, as the resulting 3D maps were not significantly different, data from sheets in both orientations were combined for each of the final maps. The comparison window for refining the phase origin was $0.08 \text{ \AA}^{-1}$ ($0.05 \text{ \AA}^{-1}$ for data from ncd-decorated sheets). The average phase residual was $18.4^\circ \pm 5^\circ$ for the tubulin data and $16.7^\circ \pm 7.5^\circ$ for data from ncd-decorated sheets. After merging the data, smooth curves were fitted using the program LATLINE²⁵ and the curves were sampled at $0.005 \text{ \AA}^{-1}$ intervals in z^* to provide the h, k, l , amplitude and phase Fourier terms for the 3D maps.

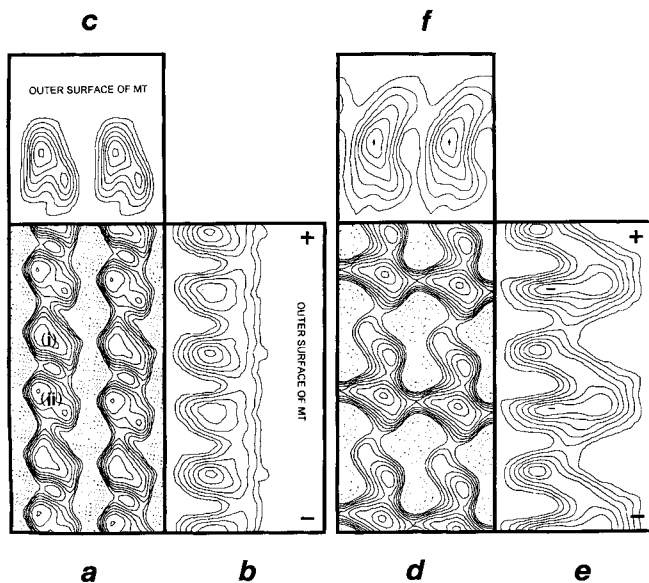
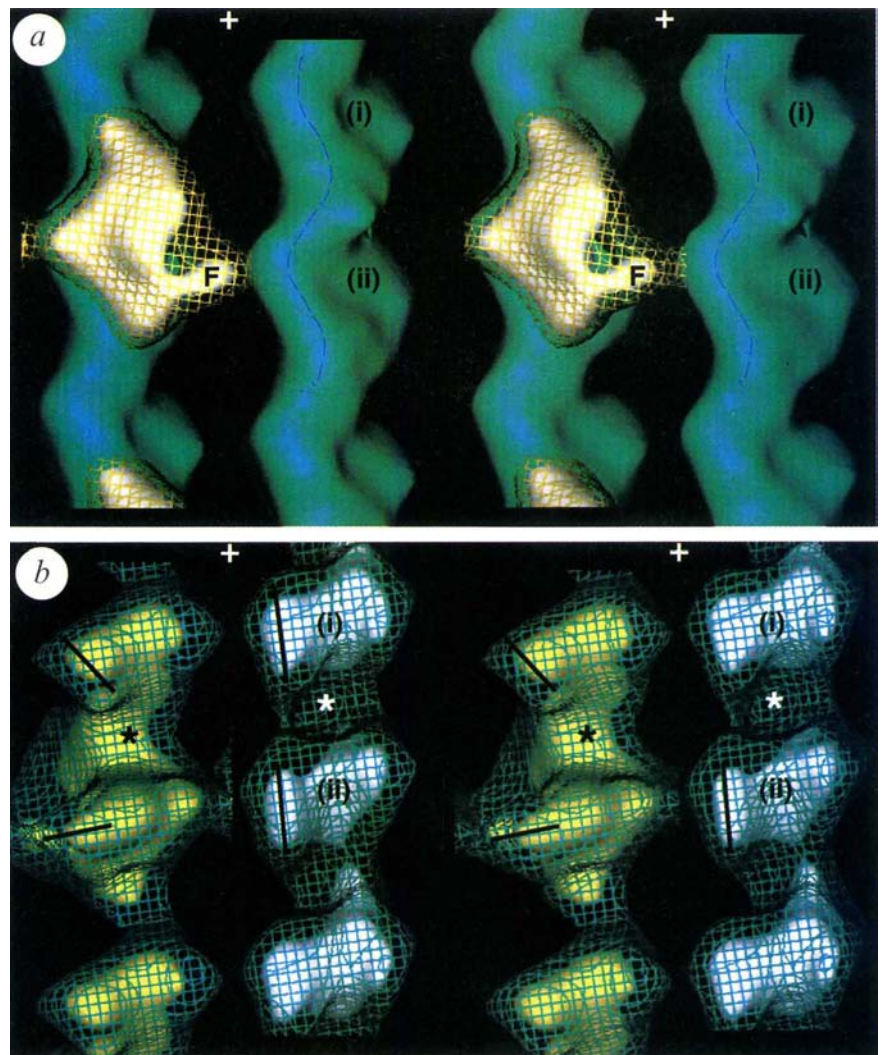


FIG. 3 Projections of the 3D maps of the tubulin sheets (a–c) and the ncd-decorated sheets (d–f). Polarity is indicated by + and –. a, d, xy projections of two protofilaments (running vertically) in the 3D map, equivalent to viewing the wall of a microtubule from the outside. b, e, Summation of the density associated with a single protofilament in the direction of the 3-start helix of a microtubule. Since the x axes in the maps and the direction of the 3-start helix are similar, we refer to b and e as zy projections for convenience. c, f, xz projections of the maps in which the density is projected along the protofilament length looking towards the plus end.

METHODS. There were no Fourier terms along the (0, 0) lattice line, so there are negative (dotted) and positive (solid) contours in the maps. The solid contours enclose stain-excluding regions, that is, regions where biological material is concentrated. The negative contours delineate regions occupied by the stain. In the z direction, strong positive and negative peaks do not overlap and all densities were summed to calculate the xy projections. However, in calculating the xz and zy projections only the positive densities were summed as there is substantial overlap of positive and negative peaks in these projection directions. Summing all densities gives results that are confusing and difficult to interpret. The sheet polarity was determined by growing sheets from the plus end of axonemal fragments^{1,2}. Images in which the relation between the sheets and the axonemal fragments was clear were analysed. 2D projection maps were calculated by averaging the data from 16 images of undecorated and 14 images of ncd-decorated sheets. The maps of known polarity were then related to the xy projections of the 3D maps.

FIG. 4 a, Structure of the ncd motor domain and the tubulin protofilament. Stereo view of two adjacent protofilaments from the 3D map of tubulin sheets (turquoise). The estimated molecular boundary of the ncd motor domain (yellow wire mesh) and an internal high-density region (white surface) are shown on the left protofilament. The finger referred to in the text is labelled F; (i) and (ii) identify the tubulin monomer type (see also Fig. 3a). The broken line follows the path of the smooth outer surface of the unlabelled protofilament. b, Stereo views of a single protofilament from each of the 3D maps. The view is equivalent to looking at the inside surface of a microtubule wall. Molecular boundaries (wire mesh) and internal high-density regions (solid colours) are shown. The map from ncd-decorated sheets is on the left (yellow internal contour) and the map of tubulin sheets is on the right (white internal contour). The long axes of domains in the tubulin monomers are indicated by short lines. The asterisk identifies a region between two monomers that is enlarged when ncd is bound.

METHODS. To create the ncd difference map, the densities in common parts of the two 3D maps (sections corresponding to the lower 75% of the tubulin molecules) were first scaled together. Then the maps were brought to the same phase origin by iterative correlation of orthogonal projections before subtracting one map from the other. Owing to the structural changes in tubulin, there are small difference peaks in the tubulin region of the map (not shown), and the difference map shown is inaccurate near the tubulin–ncd interface. As the tubulin changes were unexpected, we explored a number of possibilities that might explain the results. We truncated both data sets to identical and then progressively poorer resolutions and calculated 3D maps for comparison. Even when truncated to 40 Å axially and 25 Å laterally, the data yielded maps in which the peaks attributable to tubulin were still significantly different in the presence and absence of the motor domain. Similar results were obtained when we removed low-resolution data. These experiments demonstrate that the observed structural changes are due to differences distributed throughout the entire data set and not simply to one or two noisy lattice lines. A second possibility is that the differences are due to altered penetration of tubulin by stain when the motor domain is bound. We think that such staining differences are unlikely to account for all the observed changes because



the regions of tubulin most distant from the motor domain—regions least likely to have their staining characteristics altered by the additional mass—do show substantial differences. We therefore conclude that most of the observed differences reflect real conformational changes induced in the tubulin molecules by ncd attachment.

additional noteworthy feature of the *ncd* motor domain is the finger-like projection, attached to the top of the main part of the motor, that touches an adjacent *ncd* (Fig. 4a, F). This contact is probably not present when protofilament sheets are curved in the microtubule wall (see above). The finger is an attractive structural candidate for the linkage point to the *ncd* stalk.

One unexpected result of the comparison of the two maps is that the tubulin molecules are substantially different in decorated and undecorated sheets. The results of several tests on the data convince us that the differences are real and not due to either noise or staining artefacts (see Fig. 4 legend). Views of the maps equivalent to looking from the inside of a microtubule reveal the differences clearly (Fig. 4b). With no motor domain attached, the tubulin monomers are characteristically bilobed with the long axes of the lobes aligned roughly along the protofilament axis. This arrangement creates deep 'pockets' between the monomers (Fig. 4b, asterisk). When *ncd* is bound, the pockets are greatly enlarged. It seems that the increase in pocket size is a result of a change in orientation of the monomer lobes. The greatest change is in the type (ii) monomer, which has the major contacts to the *ncd* motor domain. The details in the map give the impression that one lobe has rotated by about 90° so that its long axis now lies perpendicular to the protofilament axis. A similar, although less pronounced, apparent rotation of the corresponding lobe of the other monomer is also seen (Fig. 4b, dark lines). In the second lobe of each monomer, there are less well defined changes in density distribution. Remarkably, the lattice periodicities are unchanged. These data suggest that the tubulin molecule has mobile structural elements overlying a stable molecular skeleton that is responsible for the intermolecular bonding in microtubules. These and other observations^{20, 22} underscore the conformational flexibility of the tubulin molecule.

The function of the structural changes in tubulin is not clear. However, the changes may be indicative of models of motor–protofilament interaction involving allosteric mechanisms. Two possibilities seem attractive. First, if the cycle of interaction involves a sequential two-step process (as suggested earlier), conformational changes induced in one tubulin monomer by motor-domain attachment could be propagated to the adjacent monomer, increasing its affinity for the second site on the motor domain. Alternatively, if propagation of a structural change is favoured in one direction and extends to the adjacent tubulin heterodimer, such a model could explain directional walking of a two-headed motor on the protofilament. □

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Three-dimensional structure of the kinesin head–microtubule complex

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KINESIN is a microtubule (MT)-associated 'motor' molecule fundamental to organelle transport^{1,2}. Recently, various kinesin superfamily members (KIFs) have also been identified and suggested as being responsible for the transport of specific organelles^{3–5}. Kinesin is a heterotetramer composed of two heavy chains and two light chains. The heavy chains form two globular heads, a rod and a fan-like tail completed by the light chains^{6,7}. The globular head, which is composed of approximately 340 amino-terminal residues of the heavy chain, includes both ATP-binding and MT-binding domains, and its recombinant protein also has these properties⁸. To improve the understanding of the mechanism of force generation by an MT-based molecular motor, kinesin, we report here the three-dimensional structure of the complex of a recombinant kinesin head and MTs, as revealed by helical reconstruction from cryo-electron micrographs. A kinesin head is a globular teardrop-like structure binding to the ridge of one protofilament of MTs. We have determined the polarity of the structure of the complex of MTs and the kinesin head in relation to MT polarity.

The MT surface has been decorated with K340 (ref. 9), which contains the 340 N-terminal residues of the mouse kinesin heavy chain¹⁰. Until now, studies of the three-dimensional structure of the kinesin head–MT complex have been limited^{9,11,12} because most MTs have a seam line along the protofilament⁹, which breaks the conventional helix. But if the core MT has 10 protofilaments, the K340–MT complex should be a single-start helix with 8 nm pitch, according to the lateral accommodation model of the MT¹³. We therefore selected 10-protofilament K340–MT complexes for study, on the basis of their moiré pattern in cryo-electron micrographs^{13,14}. As predicted, the true pitch along the filament was approximately 210 nm (Fig. 1a), and the symmetric feature was confirmed by the layer-line data in the diffraction pattern (Fig. 1c), which could be indexed as a helical pattern (Fig. 1d).

For comparison, the three-dimensional structure of a bare MT with 10 protofilaments was constructed with the same helical symmetry (Fig. 2a). This showed that the 10-protofilament MT was structurally similar to the one with 14 protofilaments in terms of the basic structure of the protofilaments¹⁵. The outer surface of the MT is smooth along the protofilament, which is consistent with the surface structure previously revealed by quick-freeze deep-etch electron microscopy^{16,17}. Although the layer-lines corresponding to 8 nm were incorporated (the diffraction of MT is not shown here), the signal from them was

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so weak^{15,18} that we could not find significant differences between α - and β -tubulins.

Next we constructed the three-dimensional structure of the K340-MT complex. A surface rendering shows the arrangement of molecules in the complex (Fig. 2b). Compared with a bare MT, K340 is easily recognized as a globular teardrop-like structure attached to the ridge of only one protofilament of a MT, with little direct interaction with the adjoining protofilaments. Along the filament axis, K340 seems to have stronger interactions with one high-density region (Fig. 2d, filled arrow) and also have weaker interactions with another (Fig. 2d, open arrow). Each high-density region should correspond to a tubulin monomer, and a crosslinking study implies that the one with the stronger interaction is β -tubulin^{9,11}. K340 itself is 5–6 nm in diameter and has two protrusions; one is indicated in Fig. 2c

(p₁, arrowheads), tilting in an anticlockwise direction as seen from the top. Another was seen hanging down along the axis (Fig. 2d, p₂ with open arrowhead). These protrusions show that the attachment of K340 to the MT has a polarity corresponding to the MT polarity.

To establish the polarity of the core MT of the complex, we used a K340-decorated tubulin sheet elongated from flagellar MT¹⁹ (Fig. 3a). The top of the figure corresponds to the plus end as judged by the end structure, and the sheet is seen with its outside toward the observer so that cross-striations run up to the left. The translationally filtered image of the sheet (Fig. 3c) and the projected image generated from the three-dimensional reconstruction (Fig. 3d, same polarity as in Fig. 2b) are shown. In both maps the protofilament density is visible running at the centre (Fig. 3c, d, broken lines) and also a high-density region stretches from the protofilament (Fig. 3c, d, arrowhead) in a direction corresponding to the K340 protrusion shown as p₁ in Fig. 2c. This shows that the tubulin sheet in Fig. 3c and the MT in Fig. 3d have the same polarity. These results indicate that the bottom end of the Fig. 2b is the minus end of the MT.

To clarify the track used by kinesin, the structure shown here provides important information. Here we found that a kinesin head binds on the ridge of one protofilament. This feature itself was predicted from the existence of the seam line on the K340-MT complex, where K340 attaches only one protofilament despite the irregular alignment of the neighbouring protofilament⁹, and the feature has been directly shown here. The question arises as to whether or not kinesin uses the ridge

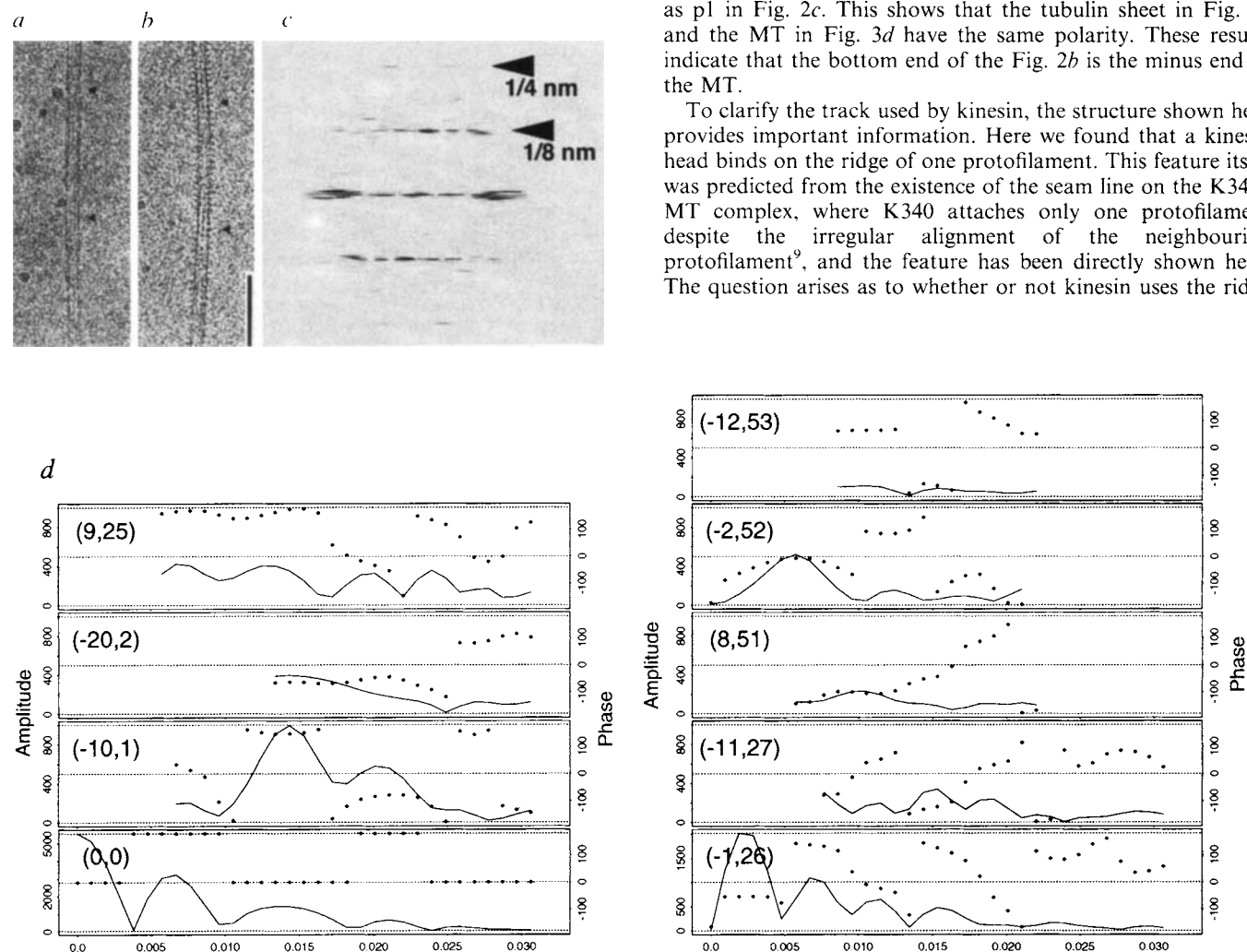


FIG. 1 Cryo-electron micrographs showing MT (a) or K340-MT complex (b), unstained and embedded in vitreous ice. Arrowheads indicate a true pitch. Scale bar, 50 nm. c, Fourier transform of b. The transform consists of a series of horizontal lines (layer-lines), each corresponding to a set of helices. Each set of helices is indexed by (n, l) on an associated surface lattice. (d) The amplitudes and phases of final averaged layer-line data of the K340-MT complex are shown with solid lines and dots, respectively.

METHODS. K340, a recombinant mouse kinesin head domain (residues 3–340) expressed in *Escherichia coli*, was prepared as described⁹. Tubulin was purified from porcine brains^{22,23}, polymerized in buffer A (MES 100 mM, pH 6.8, EGTA 1 mM, MgCl₂ 1 mM, GTP 1 mM, taxol 10 μ M) for 15 min at 37 °C, then mixed with buffer B (as A but without GTP and supplemented with 5'-adenylylimidodiphosphate (AMP-PNP)

1 mM) with 5 mg ml⁻¹ K340 at 20 °C. The specimen was put on holey carbon film and plunged into an ethane slush²⁴. Grids were examined with a Hitachi HF-2000 (200 kV) electron microscope equipped with a cold field-emission gun and Gatan cryo-transfer holder at a linear magnification of $\times 30,000$ and 2.7 μ m underfocus as described²⁵. Individual filaments on electron micrographs were digitized at a step size of 5 μ m with a charge-coupled-device film scanner (LeafScan45, Scitex). Curved filaments were straightened²⁶ and helical reconstruction was done in the standard way²⁷ using the selection rule of $l = 26n + 259m$ and 215.6 nm of true pitch¹³. The programs and libraries of MRC (Cambridge) were used with slight modification. Four helical repeats were interpolated and floated into an array of size 256 $\times$ 2,048 and Fourier-transformed, and 9 layer-lines were extracted^{28,29}. Near- and far-side layer-lines were averaged (phase residual was 47°).

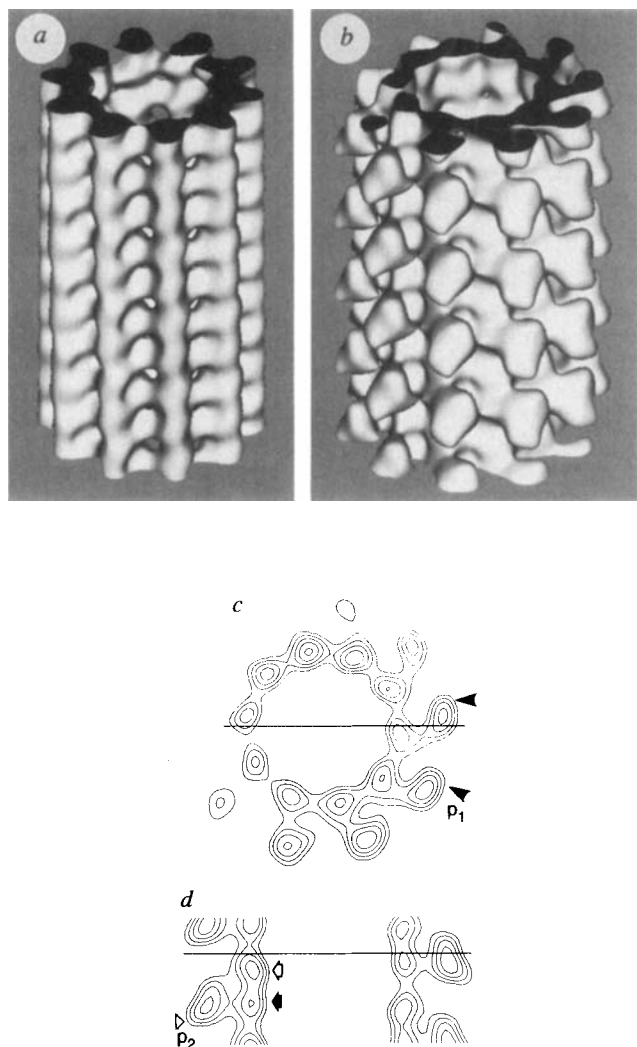


FIG. 2 Three-dimensional maps of a microtubule and a K340-MT complex. Surface representation of the computed three-dimensional maps of an MT (a), and a K340-MT complex (b); contour plot of the horizontal section (c), and vertical section (d) of the density of the K340-MT complex. Lines in c and d indicate the position of the section in a and b, respectively.

METHODS. Three-dimensional density map generated from the extracted layer-lines shown in Fig. 1 by Fourier-Bessel transformation and Fourier synthesis. The cutoff value was selected so that the volume of one unit consisting of α - and β -tubulin and the kinesin head had a relative molecular mass (M_r) of 142K, assuming the protein density to be 1.35. Surface rendering was done with ConvexAVS.

of a protofilament as a track. As noted previously, kinesin has been shown to follow the MT protofilament axis¹⁴ and the movement deviation from the microtubule axis is very small²⁰. Taken together, it is most probable that kinesin moves along a smooth ridge of a single protofilament, and does not cross the groove between protofilaments.

The use of cryo-electron microscopy has a great advantage not only in avoiding artefacts caused by staining, but also in direct visualization of the movement of molecules at the nanometre level because 'snap shots' can be taken by freezing the specimen at an intermediate state of the chemical cycle²¹. In addition, the use of 10-protofilament MT for structural studies will contribute to studies of other MT associated proteins. In particular, a deeper understanding of mechanochemical coupling should result from knowing the structure of the MT complexed with distinct members of the kinesin superfamily that move MTs

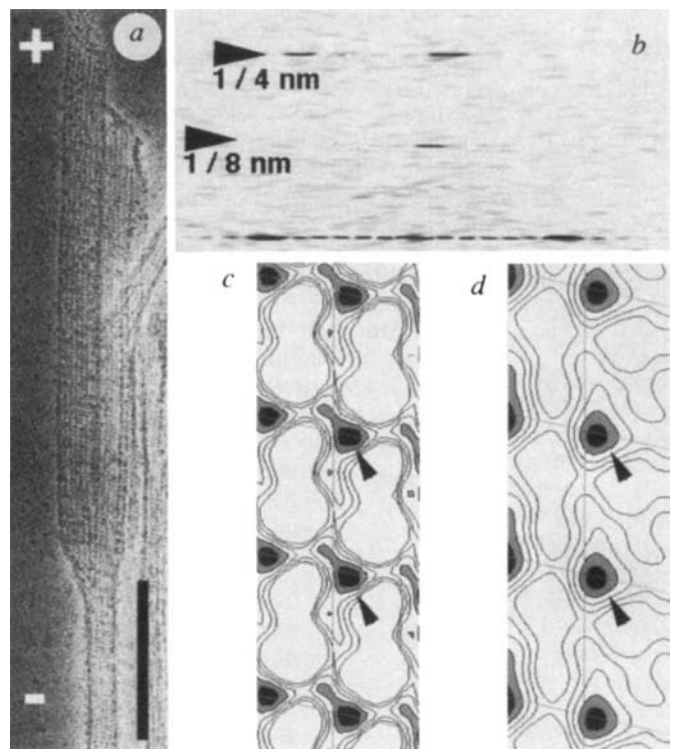


FIG. 3 Determination of the polarity of the core microtubule. a, Negatively stained tubulin sheet elongated from flagellar MTs decorated with K340. Scale bar, 50 nm. The bottom end of this figure corresponds to the compact end of the flagellum (the minus end). b, Fourier transform of the sheet region. c, The translationally filtered image of the transform, shown as a contour plot. d, Projected image of one protofilament calculated from the density map of the K340-MT complex. Vertical and oblique dashed lines in c and d indicate the centre of the protofilament and the axis of cross striation, respectively.

METHODS. Demembrated flagella axonemes were prepared from *Chlamydomonas*³⁰. K340-decorated tubulin sheet was prepared as described elsewhere¹⁹ and stained with 2% uranyl acetate and observed by JEM-1200EX and JEM-2010F (JEOL) electron microscopy at a magnification of $\times 30,000$. The sheet region was selected and the image was translationally filtered by combining only the components consistent with the translational symmetry⁹. For d, the density map of the K340-MT complex was untwisted slightly to make the protofilament and the axis parallel, and one protofilament was projected toward the centre of the tube.

in opposite directions at various speeds with two heads or with one head^{4,5}. □

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Nucleotide-dependent angular change in kinesin motor domain bound to tubulin

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KINESIN is a 'motor' molecule, consisting of two head domains, an α -helical coiled coil rod, and a tail part that binds to its cargo^{1,2}. When expressed in a bacterial system, the head domain is functional³, and can bind to microtubules with the stoichiometry of one head per tubulin dimer. Kinesin moves along microtubules by means of a cyclic process of nucleotide binding, hydrolysis and product release^{4,5}. We have used negative-stain electron microscopy and image analysis to study the structures of microtubules and tubulin sheets decorated with the motor domain (head) of kinesin in three states: in the presence of an unhydrolysable ATP analogue, 5'-adenylylimidodiphosphate (AMP-PNP); without nucleotides; and with adenosine 5'-diphosphate (ADP). A single kinesin head bound to a microtubule has a pear-shaped structure, with the broader end towards the 'plus' end of the microtubule under all conditions; the reverse motor, *ncd*, is similarly oriented. Three-dimensional maps reveal that kinesin heads have a spike that is assumed to form the attachment to the tail of a complete kinesin molecule. This spike is perpendicular to the microtubule axis in the presence of ADP, but points towards the plus end ($\sim 45^\circ$) in the presence of AMP-PNP or absence of nucleotides. Our results provide direct evidence for a conformational change of the kinesin motor domain during the ATPase cycle.

We have decorated microtubules and tubulin sheets with monomers of kinesin motor domain, K Δ 340 (the first 340 amino-terminal residues of rat kinesin heavy chain)⁶, first in the presence of AMP-PNP, which may mimic the physiological ATP or the ADP-Pi state⁴, second in the absence of nucleotide, and third in the presence of ADP. The first two are strongly bound ($K_d \sim 0.1 \mu\text{M}$) states, the third a more weakly bound ($K_d \sim 10 \mu\text{M}$) state (I. M.-T. Crevel, A.L. and R.A.C., submitted).

Microtubules and tubulin sheets can be decorated under all three conditions with a longitudinal periodicity of 8 nm, twice the spacing of tubulin monomers (Figs 1, 2). Two-dimensional computer enhancement of the periodic components shows that, in all three cases, the added protein density due to K Δ 340 seems to be spread over both monomers of each tubulin dimer but is more concentrated over one monomer, often producing the effect

of a continuous strong white line across a row of protofilaments (Fig. 1).

We have previously established the polar orientation of kinesin on microtubules in the AMP-PNP state⁷, although our results disagree with those reported by others⁸. Here, we have checked the polarity of the ADP and nucleotide-free states on sheets grown from the ends of flagellar microtubules (Fig. 1a–d). In all three states the main mass of the kinesin head is superimposed on to the tubulin monomer closer to the plus end of the microtubule, implying that, at different stages in the kinetic cycle, the motor remains bound in a similar manner to the same site on the microtubule. Sheets decorated with the motor domain of the structurally similar *Drosophila* non-claret disjunctal (*ncd*) protein, which moves in the reverse direction^{9,10}, look the same (Fig. 1e, f). Competition experiments also indicate that *ncd* and kinesin bind to overlapping sites⁶.

Nevertheless, micrographs of microtubules decorated with kinesin heads in the three states show consistent, subtle differences in appearance (Fig. 2b–g). To obtain three-dimensional images we used natural microtubules with 16 longitudinal protofilaments that twist slowly around the microtubule axis (Fig. 2e–g); their helical symmetry provides sufficient independent views in a single image to calculate a three-dimensional density map, although averaging a number of different images was required to reduce the noise to a satisfactory level. Compared with undecorated 16-protofilament microtubules¹¹, all the maps in Fig. 3 show a similar distribution of additional density on the outer surface. At the outermost radius, the extra density in all three maps ends in a spike that is curved clockwise when viewed from the plus end (see asterisks in Fig. 3).

There are small differences between different states in various parts of the three-dimensional maps but the most significant change appears to be in the angle of the projecting spike: it is tilted towards the plus end in the AMP-PNP and nucleotide-free maps (Fig. 3c, f) but is normal to the axis in the ADP map (Fig. 3i). Because it is furthest from the microtubule, it is

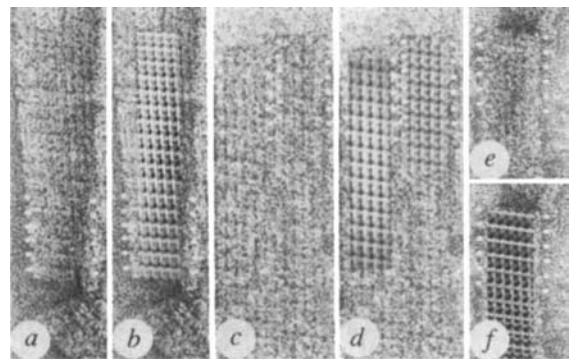


FIG. 1 Electron micrographs of tubulin sheets decorated with kinesin heads in the absence of nucleotides (a), and presence of ADP (c), or with *ncd* heads in the presence of AMP-PNP (e). Sheets are shown oriented plus end up, based on axoneme polarity⁷. The examples include a minus end (a) and 2 plus ends (c, e); c includes a 'seam' between 2 segments of a sheet whose dimers are out of phase (see examples in ref. 18). The filtered images (b, d, f) all show the kinesin or *ncd* head domain in a similar orientation and bound to the same type of tubulin monomer, as judged by the position of the motor relative to the ends of the protofilaments. Magnification: $\times 250,000$.

METHODS. Tubulin purified from brain tissue was polymerized from the ends of sea urchin axonemes and stabilized with taxol as described⁷, then incubated with 2–5 μM K Δ 340 (residues 1–340 of rat kinesin heavy chain, expressed and purified as described⁶) or N Δ 332 (residues 332–700 of *Drosophila ncd*) plus 1 mM AMP-PNP, or 1 unit per ml apyrase (Sigma grade VII: 1 unit liberates 1 μmol of inorganic phosphate per min at pH 6.5, 30 $^\circ\text{C}$) to eliminate any ADP associated with kinesin heads, or 1 mM ADP, and then negatively stained with uranyl acetate.

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assumed to be the part of the kinesin head that forms the attachment to the rod in a complete kinesin molecule. Some of the other differences between the maps may represent intramolecular rearrangements, unclear at this resolution, that cause the projection to rotate. The observed rotation seems to be reliable because the angle of the spike is consistent between individual maps of each state.

Kinesin dissociates from the microtubule at some stage after ATP hydrolysis and rebinds as kinesin-ADP (ref. 5). Thus our ADP state mimics the initial attachment state, which is followed by the tightly bound nucleotide-free state. Our maps provide evidence for an angular change of $\sim 45^\circ$ when ADP is released. The direction of the observed angular change is consistent with the plus-end directed movement of kinesin. These results support the idea that the 'power stroke' may be associated with product release¹². Binding of AMP-PNP, which may or may not mimic ATP, does not produce any further change in tilt angle, although

there seem to be further conformational changes near the kinesin/tubulin boundary (best seen in profile on the right-hand sides of the models in Fig. 3c, f).

A single kinesin head could probably not produce the 8-nm steps reported previously¹³ with a rotation of only 45° . However, if two heads of a kinesin molecule work cooperatively^{5,14,17}, a 45° change could shift the position of the second head to increase the probability of its binding to a tubulin subunit closer to the

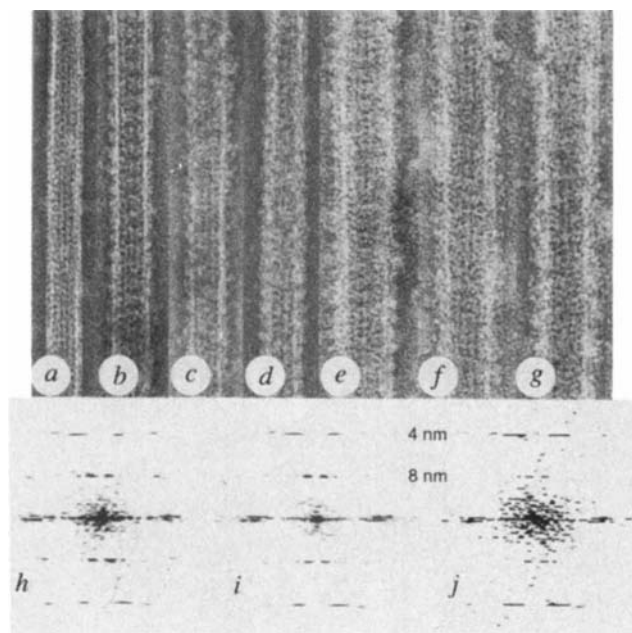


FIG. 2 Microtubules reassembled from brain tubulin (a-d), and natural, 16-protofilament microtubules (e-g); undecorated (a), decorated with K Δ 340 plus AMP-PNP (b, e), K Δ 340 without nucleotide (c, f), or K Δ 340 plus ADP (d, g). Additional density projecting from the microtubule wall with an 8-nm periodicity is the clearest with AMP-PNP, whereas the nucleotide-free decorated microtubules look smoother. With ADP, the periodic projections look rounder. h-j Diffraction amplitudes computed from images of 16-protofilament microtubules approximately twice as long as those illustrated in e-g (that is, about 2 longitudinal repeats). Because the protofilaments wind slowly around the axis (pitch $\sim 3.76 \mu\text{m}$), rather than being strictly longitudinal as in a 13-protofilament microtubule, different contributions to the nominal layer-lines ($l=0, 1, 2$) are separated vertically by $(1/235) \text{ nm}^{-1}$, a value similar to that observed for 16-protofilament brain microtubules¹⁹. The main contributions to the equator are orders $-16, 0$ and $+16$; to the 8-nm layer-line, orders $-18, -2$ and $+14$; to the 4-nm layer-line, orders $-20, -4$ and $+12$. The relative weakness of the 8-nm layer-line in ADP patterns reflects the greater disorder of the kinesin decoration in this state and/or a smaller number of bound heads.

METHODS. Microtubules polymerized from brain tubulin and stabilized with taxol were centrifuged and resuspended in a GTP-free buffer. 16-protofilament microtubules were isolated from testicular sperm of *Acheta domesticus* (ref. 20; K.H., W. B. Amos and L.A.A., in preparation) in an extracting solution (5 mM PIPES, 0.5 mM EGTA, 2 mM MgSO_4 , 1 mM DTT, 10 μM taxol, pH 7.0). Microtubules were incubated with kinesin heads as in Fig. 1. Digitized images of slightly curved microtubules were straightened using a spline-fitting routine²¹.

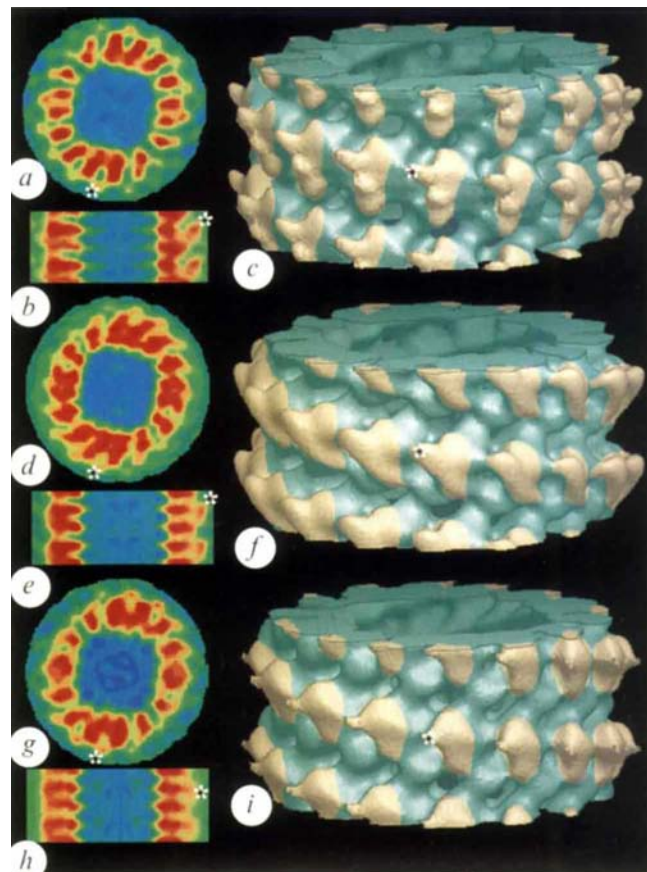


FIG. 3 Structure of microtubules decorated with kinesin heads in the presence of AMP-PNP (a-c), no nucleotides (d-f), and ADP (g-i). Horizontal sections (a, d, g), vertical sections (b, e, h), and surface representations (c, f, i). In the sections, higher protein density is represented by red; lower density by blue. The horizontal sections are viewed from the microtubule plus end, other views are placed so that the top of the picture is the plus end. Each horizontal section cuts through tubulin dimers at 8 different levels. At some levels there are separate peaks between protofilaments (see asterisks in a, d, g); at other levels, the extra density appears simply as a curved extension from each protofilament surface. The left and right halves of b, e, h are each from different vertical sections, separated by a 12° rotation. The kinesin head extends over both monomers of the tubulin dimer. In the surface maps (c, f, i), density on the outer surface that can be identified as K Δ 340, by comparison with undecorated microtubules, has been coloured cream. The precise boundary between tubulin and kinesin is unclear at this resolution. All three models include averaged data to the same nominal resolution (3.5 nm) but the AMP-PNP structure shows the most detail because microtubules decorated under this condition are the most highly ordered. A slight twist in the natural protofilaments has been omitted to simplify the computation.

METHODS. Data along the layerlines in Fig. 2 were extracted from each computed Fourier transform and processed by conventional methods^{22,23}. For each type of specimen, data from different images were closely compared and the 5-7 selected sets were averaged. Surface representations were calculated using programs written originally by G. Vigers²⁴ and processed in Adobe Photoshop.

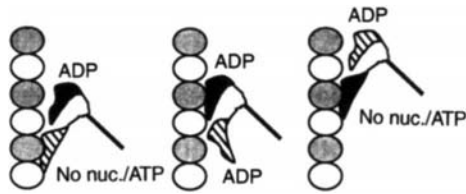


FIG. 4 A model, based on the observed change in angle, of how a pair of kinesin heads could move 8 nm along a tubulin protofilament. Although the spikes that project from the heads in our reconstructed images appear short, the extra protein sequence required to form dimers²⁵ presumably increases the leverage of the spikes. The simplest way that heads might act alternately over a long distance is for flexible connections to allow them to rotate freely around each other; the direction of movement is determined if, after the attached head changes conformation during ADP release, only the next binding site along the protofilament in the plus direction is within reach of the second head. This model is similar to model A proposed by Hackney¹⁵, except that the angle change accompanies ADP release instead of ATP binding. The kinesin-like protein *ncd* moves towards microtubule minus ends. If *ncd* has a similar projecting spike which is tilted in the opposite direction in the strongly bound no-nucleotide and AMP-PNP states, then motility in the opposite direction could be explained by a similar model.

plus end, and thus produce the 8-nm step (Fig. 4). Therefore our results are consistent with a mechanism in which structural changes in the heads result in directional movement. □

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CORRECTIONS

Structure of a new nucleic-acid-binding motif in eukaryotic transcriptional elongation factor TFIIS

Xiuqu Qian, ChoonJu Jeon, HoSup Yoon, Kan Agarwal & Michael A. Weiss

Nature **365**, 277–279 (1993)

WE previously reported binding of TFIIS residues 231–280 to single-stranded DNA by gel mobility-shift assay (Fig. 1 of this Letter). We have since discovered that the extended form (residues 175–280), rather than the shorter form (residues 231–280), of the protein was inadvertently used in this assay by one of our laboratories (K.A.). The binding of the shorter polypeptide to single-stranded DNA is not reproducible: we therefore retract Fig. 1. Although there is no published spectrofluorometric evidence of interaction between isolated zinc ribbon and nucleic acid, when site-directed mutations in the zinc ribbon domain of the intact protein are used in a stalled transcription assay this domain appears to interact with nucleic acids, as indicated by decrease or elimination of antitermination and RNA cleavage activities¹. □

1. Jeon, C. J., Yoon, H. S. & Agarwal, K. *Proc. natn. Acad. Sci. U.S.A.* **91**, 9106–9110 (1994).

Primary production required to sustain global fisheries

D. Pauly & V. Christensen

Nature **374**, 255–257 (1995)

THERE were several numerical errors published in this Letter, some kindly brought to our attention by M. Baumann and T. R. Parsons (personal communication).

■ In Fig. 1, the catch of Nile perch should be $0.01 \text{ t km}^{-2} \text{ yr}^{-1}$ (not $0.1 \text{ t km}^{-2} \text{ yr}^{-1}$).

■ In Table 1, the primary production required (PPR) for squid in the upwelling system should be 4.1, that for shrimps on tropical shelves should be 3.5, and the *k* value of miscellaneous teleosts should be 155.

■ In Fig. 2, the abscissa should change in steps of 4%.

None of these errors affects the results of our study. □

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